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Studies on epigenetic reprogramming of germ cells in mice

(マウス生殖細胞におけるエピジェネティックリプログラミングに関する研究)

Keisuke AOSHIMA

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Abbreviations

B6:	C57BL/6
BSA:	Bovine serum albumin
ChIP-seq:	Chromatin immunoprecipitation sequencing
CHX:	Cycloheximide
DAPI:	4',6-diamidino-2-phenylindole
DBTMEE:	Database of Transcriptome in Mouse Early Embryos
D-MEM:	Dulbecco's modified eagle medium
EGFP:	Enhanced green fluorescent protein
ESCs:	Embryonic stem cells
EU:	5-ethynyl uridine
FBS:	Fetal bovine serum
FGF2:	Fibrous growth factor 2
FPKM:	Fragments per kilobase of transcript per million mapped fragments
GDNF:	Glia cell line-derived neurotrophic factor
GENA:	Germ cell-specific antigen
GFRA1:	GDNF family receptor alpha 1
GSCs:	Germ stem cells
H3K27ac:	Acetylated H3K27
H3K4me1:	Monomethylated histone H3 lysine 4
H3K4me2:	Dimethylated histone H3 lysine 9
H3K4me3:	Trimethylated histone H3 lysine 4
H3K9me2:	Dimethylated histone H3 lysine 9
H3K9me3:	Trimethylated histone H3 lysine 9
H3K27me3:	Trimethylated histone H3 lysine 27
H3K36me3:	Trimethylated histone H3 lysine 36
HA:	Hemagglutinin
HBSS:	Hanks' balanced salt solutions
hCG:	human chorionic gonadotropin
HTF:	Human tubal fluid
ICSI:	Intracytoplasmic sperm injection
iPS cells:	Induced pluripotent stem cells
IU:	International unit
IVF:	<i>In vitro</i> fertilization
IVT:	<i>In vitro</i> transcription

KSOM:	Potassium simplex optimization medium
KSR:	Knockout serum replacement
MEFs:	Mouse embryonic fibroblasts
MEM α :	Minimum essential media alpha medium
PBS:	Phosphate-buffered saline
PBST:	PBS containing 0.05% Triton X-100
PFA:	Paraformaldehyde
PGCs:	Primordial germ cells
PMSG:	Pregnant mare's serum gonadotropin
PN:	Pronucleus
PNs:	Pronuclei
RNAP II:	RNA polymerase II
Rps7:	Ribosomal protein S7
RT:	Room temperature
RT-qPCR:	Reverse transcription quantitative polymerase chain reaction
SD:	Standard deviation
SEM:	Standard error of the mean
SET:	Su(var)3-9, Ez and Trx-like
SSCs:	Spermatogonial stem cells
STOs:	SIM mouse embryo-derived thioguanine- and ouabain-resistant fibroblasts
TBST:	Tris buffered saline containing 0.05% Triton X-100
TIF1 α :	Transcription intermediary factor 1 alpha
TYH medium:	Toyoda-Yokoyama-Hoshi medium
WT:	Wild type
ZGA:	Zygotic gene activation

Notes

Contents of the present thesis were published in the following articles.

1. **Aoshima K, Inoue E, Sawa H, Okada Y.** 2015. Paternal H3K4 methylation is required for minor zygotic gene activation and early mouse embryonic development. *EMBO Rep.* **16**:803-12.
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2. **Aoshima K, Baba A, Makino Y, Okada Y.** 2013. Establishment of Alternative Culture Method for Spermatogonial Stem Cells Using Knockout Serum Replacement. *PLoS One.* **8**:e77715.
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General Introduction

Epigenetic reprogramming is a process through which epigenetic modifications are dynamically altered to change the cellular state from differentiated to undifferentiated. It naturally occurs to produce undifferentiated cells such as zygotes and primordial germ cells (PGCs) during mammalian embryonic development [1, 2]. Artificially, it has been first demonstrated by the nuclear transfer to unfertilized oocytes in *Xenopus laevis* by Gurdon J *et al* in 1958 [3], and Campbell KH *et al* also demonstrated it by creating cloned sheep with same procedure in 1996 [4]. Recently, Takahashi and Yamanaka has demonstrated reprogramming *in vitro* by transducing defined genes (Yamanaka factors: *Oct4*, *Sox2*, *Klf4* and *c-Myc*) into somatic fibroblasts and named the cells induced pluripotent stem cells (iPS cells) [5]. Revealing the molecular mechanism of reprogramming can confer significant benefits for basic sciences such as molecular biology and embryology, and applied sciences such as fertilization treatment and regenerative medicines. Regardless many efforts to elucidate the molecular mechanism, the whole picture is not clear.

Epigenetic modifications such as DNA methylation and histone modifications can regulate gene expression without changing the genomic sequences. DNA methylation on promoter regions can repress the gene expression, whereas histone methylation can regulate gene expression actively or repressively depending on the methylated site [6, 7]. Since epigenetic modifications are inherited to daughter cells and keep the same gene expression profiles as mother cells, tissues can maintain their function and structure by producing same type of cells [8, 9]. During differentiation, however, cells change their gene expression profiles to obtain unique functional phenotypes [10-13]. Therefore, differentiated cells have distinct epigenetic properties according to each cell type because the change of gene expressions is accomplished by dynamic alteration of epigenetic modifications [14-16]. In reprogramming, the epigenetic properties are significantly altered to reset their

differentiated phenotype and obtain totipotency or pluripotency [1, 17, 18].

Although reprogramming can be achieved by artificially, the success rate is significantly lower than that of natural reprogramming in zygotes and PGCs. Natural zygote can obtain totipotency and become individuals in higher probability compared to cloned embryos, and, on top of that, reprogramming efficiency of fibroblasts by Yamanaka factors are below 1% [4, 5, 19, 20]. As the reason why the difference between natural and artificial reprogramming comes up is still unclear, I studied the molecular mechanisms of natural reprogramming of mice germ cells to find the key of effective reprogramming.

Chapter 1

Paternal H3K4 methylation is required for minor zygotic gene activation and early mouse embryonic development

Introduction

Zygotes are the only cells that have totipotency, which is the ability that cells can differentiate into all type of cells. Although zygotes are derived from sperms and oocytes, both of them do not have totipotency and cannot differentiate any more. Therefore reprogramming is required for zygotes to establish totipotency soon after fertilization. In zygotes, it occurs at the pronuclear stage, during which zygotes have two distinct nuclei that are derived from the sperm [paternal pronucleus (PN)] and the oocyte (maternal PN). The pronuclear stage is subdivided into five substages (PN1-5) according to the time after fertilization and the locations of the pronuclei (PNs) (Fig.1) [21, 22]. In the PNs, genome-wide epigenetic alteration predominantly occurs in the paternal PN rather than the maternal PN, implying that paternal epigenetic reprogramming is more dynamic than maternal reprogramming [21-27]. For instance, recent studies have demonstrated that the genome-wide active DNA demethylation is dependent on Tet3, a member of the DNA demethylase family [28], while maternal DNA methylation is protected from active DNA demethylation by the binding of PGC7 to dimethylated histone H3 lysine 9 (H3K9me2), which the paternal PN lacks [29]. Like H3K9me2, many histone modifications in the paternal PN are unestablished or gradually become detectable as the pronuclear stage progresses. In contrast, there is no such dynamic alteration of histone modification in the maternal pronucleus. In addition to histone modifications, localization of histone variants is also asymmetric between the paternal and maternal PNs. Histone H3.3, one of the histone variants that is incorporated into chromatin independently of DNA replication, is predominantly incorporated into the paternal pronucleus soon after fertilization [25, 30]. The impairment of K27 methylation and K36 methylation of H3.3 by using their lysine-arginine substituted mutants (i.e., H3.3-K27R and H3.3-K36R, respectively) has been reported to critically affect the development of preimplantation embryos; these effects were not

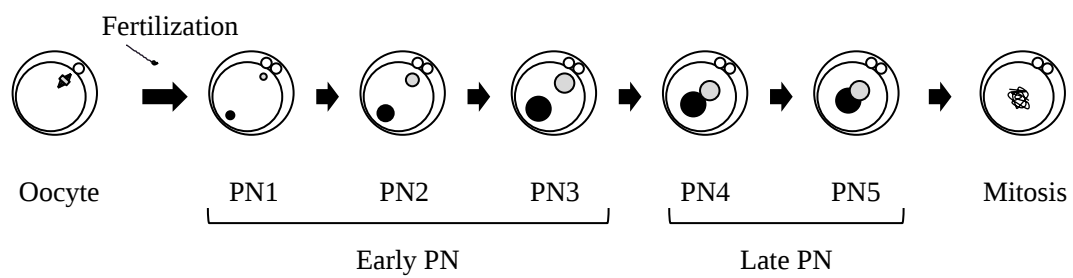


Fig.1 Images of substages in the mouse pronuclear stage.

The pronuclear stage is subdivided into five substages. PN1-3 and PN4-5 are also called early PN and late PN, respectively.

observed when H3.1 was used [26, 31]. Collectively, these findings demonstrate the critical role of histone methylation in embryonic development,

Immediately after fertilization, maternal factors, including RNAs and proteins, are abundantly retained in zygotes. During pronuclear stages, these maternally derived components degrade rapidly, and transcription of the zygotic genome has to be initiated for subsequent embryonic development [32, 33]. Gene transcription during the transition from maternal factors to those of the zygote is called zygotic gene activation (ZGA), which consists of two waves: the late pronuclear stage (minor ZGA) followed by the two-cell stage (major ZGA) [34, 35]. Minor ZGA is the first activation of transcription from the zygotic genome, and occurs at the start of the late pronuclear stages. Interestingly, it has been reported that the timing and global gene expression levels are different between the paternal and maternal PN: the paternal PN exhibits earlier and higher gene transcription compared with the maternal PN [36]. The reason for this transcriptional asymmetry is thought to be differences in their chromatin structures. However, exactly what kind of epigenetic factor(s) causes the asymmetric transcription in minor ZGA has not yet been elucidated.

An enhancer is a *cis*-regulatory element that activates gene expression and is primarily involved in the establishment of cell-specific gene expression [37]. Enhancers have characteristic epigenetic properties, which include monomethylated H3K4 (H3K4me1) and acetylated H3K27 acetylation in active genes, and H3K4me1 and H3K27me3 in poised genes [38-40]. *Mll3* (also known as Kmt2c) and its paralog *Mll4* (also known as Kmt2d), both of which are H3K4 methyltransferases, mediate H3K4me1 at enhancer or promoter regions and have partial functional redundancy [41, 42]. *Trr*, a *Drosophila* homolog of *Mll3/4*, mediates H3K4me1 at enhancer regions, and the loss of *Trr* decreases global H3K4me1 *in vivo* [43, 44]. Further, Lee *et al.* have shown that *Mll4* is crucial for enhancer activation during cell differentiation using adipogenesis and myogenesis as a model system [45]. Although the *Mll3* protein has been detected in mouse MII oocytes by

proteomic analysis [46], its function and contribution to H3K4me1 for epigenetic reprogramming in zygote remain elusive.

Recently, a search for *de novo* mutations in paediatric glioma patients identified a genetic mutation of lysine 27 to methionine (K27M) in the histone H3.3 gene [47]. Interestingly, this mutation not only abrogates K27 methylation in the H3.3 transcribed from the mutated genome, but also inhibits global endogenous K27 methylation in non-mutated H3, including H3.1 and H3.2, by preventing the methyltransferase activity of PRC2. In addition, the mechanism that prevents catalytic activity is often similar to other Su(var)3-9, Ez and Trx-like (SET) domain-containing methyltransferases; accordingly, K4M, K9M, and K36M mutants are able to inhibit endogenous K4, K9, and K36 methylation, respectively, when they are overexpressed.

To take advantage of this system, K-M mutants were employed to clarify the roles of histone methylation in ZGA. It was found that H3K4 methylation is critical for minor ZGA in the paternal PN, and is essential for early preimplantation development in mice. In addition, knockdown of *Mll3/4* in zygotes elicits a similar effect to the overexpression of the K4M mutant on the development of preimplantation embryos, indicative of the importance of H3K4 methylation for enhancer regions.

Materials and methods

Ethics statement

All experimental procedures involving animals were approved by the Animal Experiment Ethics Committees at the Institute of Molecular and Cellular Biosciences, The University of Tokyo (#23015, #2509).

Oocyte preparation and cell culture

Four-to-eight-week old BDF1 mice (C57BL/6 x DBA/2 strain, CLEA Japan, Tokyo, Japan) were used for all experiments except for GV injection. MII oocytes, collected from female mice treated with 7.5 International unit (IU) pregnant mare's serum gonadotropin (PMSG; ASKA Pharmaceutical, Tokyo, Japan) and 7.5 IU human chorionic gonadotropin (hCG; ASKA Pharmaceutical), were cultured in human tubal fluid (HTF) medium (Irvine Scientific, CA, USA) with 3 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, MO, USA). For harvesting GV oocytes, six-to-eight weeks old C57BL/6 x A129 mice were used, because it was found that the survival rate after injection was higher than for BDF1 oocytes (data not shown). Forty-six hours after PMSG injection, GV oocytes were collected from ovaries by puncturing antral follicles, and then cultured with TaM medium a Hepes-buffered 1:1 mixture of Toyoda-Yokoyama-Hoshi medium (TYH medium; LSI medience, Tokyo, Japan) and minimum essential media alpha medium (MEM α ; Thermo Fisher Scientific, MA, USA) supplemented with 5% fetal bovine serum (FBS; Biofill Australia Pty. Ltd. Victoria, Australia) and 2 mM L-carnitine (Lonza, Basel, Switzerland); termed mTaM medium [48]. 293T cells were cultured in Dulbecco's modified eagle medium (D-MEM; Wako, Osaka, Japan) supplemented with 10% FBS (Biofill), GlutaMAX-I (Thermo Fisher Scientific), and penicillin/streptomycin (Thermo Fisher Scientific). Cells were maintained at 37 °C

with 5% CO₂ before use in experiments.

***In vitro* fertilization, cycloheximide treatment, parthenogenetic activation, and natural mating**

In vitro fertilization (IVF) was performed by adding 2×10^5 sperm to the MII oocytes in HTF medium. After 4 hours, fertilized oocytes were washed by pipetting with mouth pipettes in HTF medium and cultured at 37 °C with 5% CO₂. Cycloheximide (Sigma Aldrich) was added to both IVF and embryo culture drops at a concentration of 50 µg/ml. The following day, 2-cell embryos were transferred to potassium simplex optimization medium (KSOM; ARK Resource, Kumamoto, Japan) and cultured at 37 °C with 5% CO₂ until they became blastocysts. Parthenogenetic activation of oocytes was performed by treatment with 7% ethanol for 5 minutes at room temperature (RT) [49]. Natural mating was performed by mating male and female mice treated with PMSG and hCG, and zygotes were collected from female mice outfitted with copulation plugs on the next day.

Plasmid construction and *in vitro* transcription

The cDNA of Flag-HA-tagged H3.1 and H3.3 were obtained from pOZ-e-H3.1 and e-H3.3, respectively [50]. These cDNAs were subcloned into a pcDNA3.1-poly(A)83 vector [51] and used for transfection or *in vitro* transcription (IVT). Transfection of plasmids into 293T cells was performed using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions in D-MEM supplemented with 10% FBS and GlutaMAX-I. IVT was performed using the RiboMAX Large Scale RNA Production System T7 (Promega, WI, USA) according to the manufacturer's instructions. Synthesized mRNAs were purified with Illustra MicroSpin G-25 columns (GE Healthcare, Little Chalfont, UK) before microinjection.

Microinjection of mRNAs and siRNAs and intracytoplasmic sperm injection (ICSI)

Microinjection of mRNAs and siRNAs into MI and MII oocytes or zygotes was performed using a pneumatic microinjector (IM-11-2 and IM-12, NARISHIGE, Tokyo, Japan) and a Piezo Impact Micro Manipulator (PMM-150FU, PRIME TECH, Tokyo, Japan). Approximately 3–5 pl (= polar body size) of 40 ng/μl mRNAs or 90 μM siRNAs (Thermo Fisher Scientific) were microinjected into the cytoplasm of MI and MII oocytes and zygotes in M2 medium (ARK Resource). MI oocytes, developed from GV oocytes by approximately 2 hours culture in mTaM medium, were subject to siRNA injection followed by culture with mTaM medium for 28–30 hours, and the cells that successfully matured to MII oocytes were subject to ICSI. When MII oocytes were used initially, ICSI was performed 4 hours after mRNA microinjection or 8 hours after siRNA microinjection.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

cDNA was extracted from MII oocytes, zygotes, and 2-cell embryos using SuperScriptIII Cells Direct cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Sample preparation was performed using KAPA SYBR FAST qPCR Kit Master Mix (2x) ABI Prism (KAPA Biosystems, MA, USA) or KAPA PROBE FAST ABI Prism 2x qPCR Master Mix (KAPA Biosystems), and analyzed by either an Eco Real PCR System (Illumina, CA, USA) or StepOnePlus (Thermo Fisher Scientific). Results were normalized based on the geometric average of reference genes (*Ppia*, *H2afz*, *Hprt1*) [52, 53] as a standard. For single-cell RT-qPCR analysis, *Hprt1* was used as an internal control, the C_q value of which did not vary greater than 2-fold among all samples. The average *Venus* C_q value from RT-qPCR samples was considered to be derived from a single copy of paternal genomic DNA: thus, *Venus* C_q values more than the average were calculated as 0 (= no amplification). Primer sequences for qPCR are listed in Table 1.

Primer	Sequence	
	Forward (5'→3')	Reverse (5'→3')
Setd1a	ggtgtaccgctatgacggag	gggaggggaaaagtctctggc
Setd1b	tttgccatggagccaattgc	atgtagctgctaccgatgcc
Mll1	tcaaccactctcgaatggc	ggacagagggtcaggatccct
Mll2	ctcgccactgaggatacgtc	ccctgtagaccctgtaggct
Mll3	actggagagctgaatgcacc	ctctagcagcatacaggccc
Mll4	ctgacaaagccccctacctg	cggtcgatcagtcttacggg
Ppia	cgcgtctccttcgagctgttg	tgtaaagtcaccaccctggcacat
H2afz	acagcgcagccatcctggagta	ttcccgatcagcgatttgtgga
Hprt1	gcttgctgggtgaaaaggacctcgaag	ccctgaagtactcattatagtaagggcat
Venus	ggcacaagctggagtacaacta	ggatcttgaagtggccttgat

Table1 Primer sequences for RT-qPCR

Immunostaining

Embryos were washed with phosphate-buffered saline (PBS) containing 0.1% BSA (PBS/BSA) and treated Tyrode's solution (Sigma Aldrich) to remove the zona pellucida. Denuded embryos or 293T cells were fixed with 4% paraformaldehyde (PFA) or methanol at RT for 15 minutes or at 4 °C overnight. They were then washed with PBS/BSA or PBS and permeabilized with 0.5% Triton X-100 at RT for 15 minutes. After washing with PBS containing 0.05% Triton X-100 (PBST) and blocking with 5% goat serum at RT for 1 hour, cells were incubated with anti-H3K4me3 (ab8580, Abcam, Cambridge, UK,), anti-H3K4me1 (ab8895, Abcam), anti-H3K9me3 (ab8898, Abcam), anti-H3K27me3 (07-449, Merck KGaA, Darmstadt, Germany), anti-H3K36me3 (ab9050, Abcam), anti-FLAG (Sigma-Aldrich), anti-RNA polymerase II C-terminal domain repeat (8WG16, Abcam) or anti-Brg-I (sc-10768, Santa Cruz Biotechnology, Heidelberg, Germany) antibodies in 5% goat serum at RT for 1 hour. Anti-acetylated H3K27 antibody was kindly provided by Dr. H. Kimura at Tokyo Institute of Technology, Japan. Embryos and 293T cells were then washed with PBST and incubated with Alexa Fluor 488 (Thermo Fisher Scientific) and Alexa Fluor 594 (Thermo Fisher Scientific) in Tris buffered saline containing 0.05% Triton X-100 (TBST) at RT for 1 hour followed by 4',6-diamidino-2-phenylindole (DAPI) staining (VECTASHIELD for embryo; VECTORS, CA, USA, or DAPI solution for 293T; Dojindo, Kumamoto, Japan). After washing with PBS, all samples in each experiment were collected in the same drop on a glass bottom dish, then fluorescent images were captured with confocal microscopes at the same time (CV1000, Yokogawa Electric Corporation, Tokyo, Japan or LSM780, Carl Zeiss AG, Jena, Germany) and processed with ImageJ software [54-56]. Results were obtained from at least three independent experiments with total sample size >10.

Global transcription assay

Global transcription assays were performed using a Click-iT RNA Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, zygotes were incubated with 2 mM 5-ethynyl uridine (EU) at 37 °C for 2 hour, treated with Tyrode's solution, fixed with 4% PFA, and treated with methanol for PN zygotes at -30 °C for 10 minutes or 0.5% Triton X-100 for 2-cell embryos at RT for 15 minutes. After washing with TBST three times at RT, embryos were then incubated in the Click-iT reaction cocktail at RT for 30 minutes and then washed with the Click-iT reaction rinse buffer. After DNA staining with VECTASHIELD (VECTOR), fluorescence images were captured using a CV1000 microscope and processed with ImageJ software. Results were obtained from at least three independent experiments with total sample size >10.

Data quantification and statistical analysis

Images of each 2 µm section were obtained by CV1000 and intensities of (pro)nuclei and cytosol (equal size to the nucleus for comparison) for each section were measured by manually outlining each (pro)nucleus with ImageJ software after background subtraction. When it was difficult to find the nuclear region due to very low intensities, signals of FLAG, H3K27ac or DAPI were used for guides. After calculating total intensities of each whole (pro)nucleus and cytosol by summing the intensities of all of its sections, relative intensities (i.e., ratio of paternal/maternal PN for zygote, and ratio of nucleus/cytosol for 2-cell embryo) were calculated. Statistical analysis for embryonic survival rates were performed using the chi-square test [57, 58]. For the comparison of the expression levels of *Mil3* and *Mil4* after siRNA treatment, Student's *t* test was performed. For other experiments, the Shapiro–Wilk test was first used to check whether the data was normally distributed or not. When data was normally distributed, Bartlett's test was used to check for equality of variance, whereupon ANOVA and Tukey–Kramer test were performed for data having equal

variance and the Games–Howell test for data having unequal variance. When data was not normally distributed or at least one group in the data was not normally distributed, the Steel–Dwass test was performed.

Results

H3K4 methylation of the paternal pronucleus is required for early preimplantation development

To verify the role of histone methylation in preimplantation embryos, K-M mutants against lysine 4 (K4M), 9 (K9M), 27 (K27M) and 36 (K36M) residues in histone H3.1 or H3.3, respectively, were prepared. H3.3 was preferentially selected instead of H3.1 or H3.2, because H3.3 is rapidly incorporated into chromatin at the early pronuclear stage after fertilization, whereas the incorporation of H3.1/H3.2 is markedly delayed [59]. Indeed, several studies have documented the importance of H3.3 over H3.1 in early embryonic development [25, 30]. Global inhibitory effects of histone methylation by overexpressing these K-M mutants were first confirmed in 293T cells (Fig. 2).

For mouse embryos, overexpression was induced by injecting the mRNAs of these mutants into the cytoplasm of MII oocytes followed by intracytoplasmic sperm injection (ICSI), which ensured the translation of mutant proteins from the injected mRNAs by the time of fertilization and early zygotic stages (Fig. 3A, red line). mRNA injection after *in vitro* fertilization (IVF) was also performed, in which the mutant proteins were expressed from the late pronuclear stages (PN4–5) onwards (Fig. 3A, blue line). In both experiments, embryonic development was monitored until the blastocyst stage. It was found that injection of H3.3 K4M mutants before fertilization significantly decreased the embryo survival rate (Fig. 3B). Interestingly, this effect was not observed in the case of injection after fertilization (Fig. 3C), suggesting that although the retarded cell growth in H3.3 K4M-injected embryos occurred after the 8-cell stage (Fig. 3B), the cause of the abnormality had already occurred in the zygotic stages (Fig. 3A). Other than the H3.3 K4M, the H3.3 K36M also exhibited significant growth retardation when it was

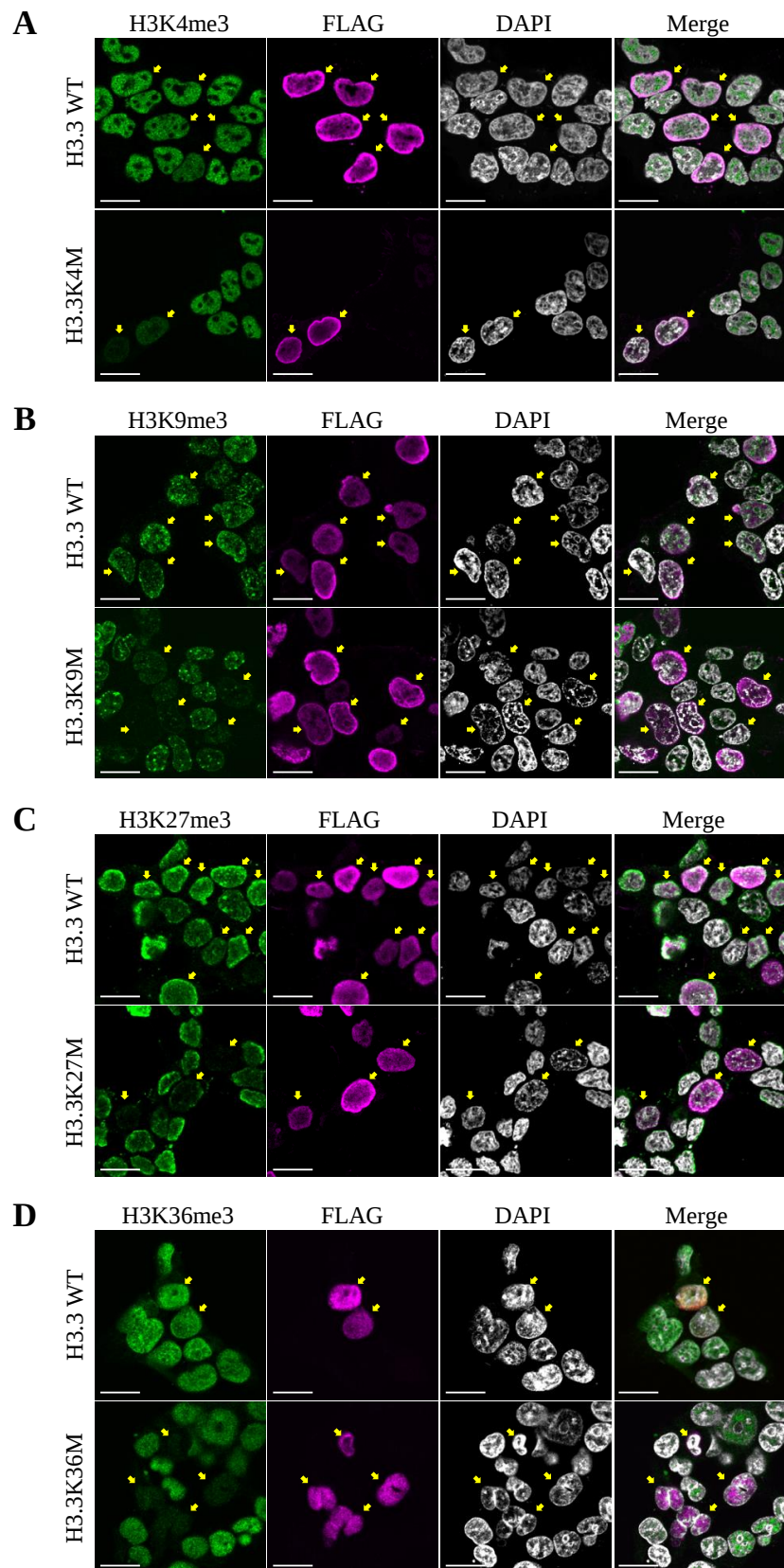


Fig. 2 K-M mutants decrease global endogenous histone methylation.

Immunofluorescent images of 293T cells overexpressed K-M mutants: H3.3 K4M (A), H3.3 K9M (B), H3.3 K27M (C) and H3.3 K36M (D), respectively. Arrows indicated the cells in which H3.3 wild type (WT) or each K-M mutants were overexpressed. Bars = 20 μ m.

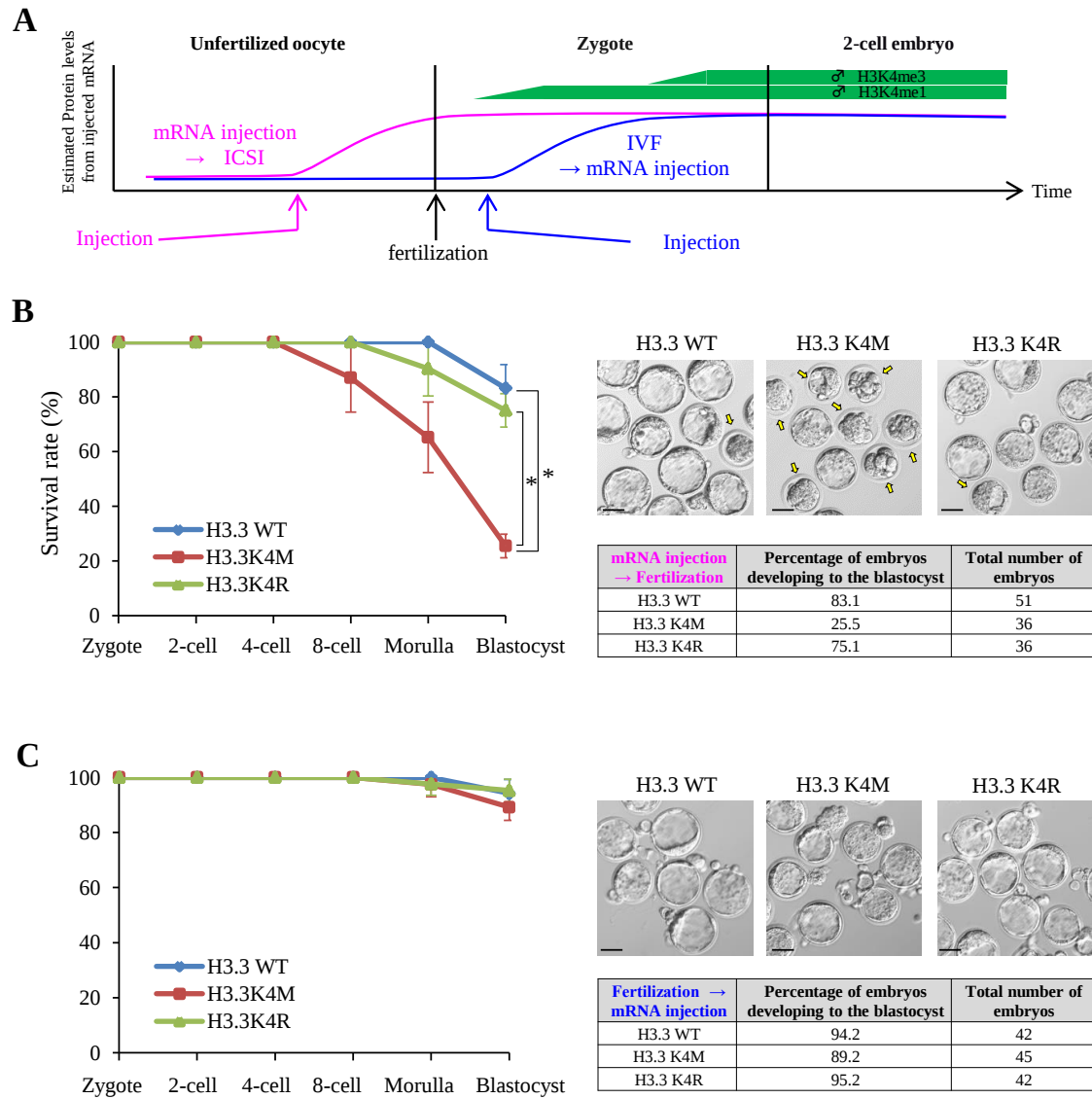


Fig. 3 Overexpression of H3.3 K4M before fertilization causes the developmental arrest of early preimplantation embryos.

(A) A scheme of the experimental procedure. Red and blue lines indicates the estimated protein levels from injected mRNA injected prior to and after fertilization, respectively. Green bars indicated H3K4me1/3 modification detected in paternal genome. (B, C) Developmental rate of zygotes overexpressing H3.3 K4M or H3.3 K4R before (B) or after (C) fertilization. Each figure shows survival curves (left), representative images at the blastocyst stages (right top), and developmental rates (right bottom). Survival curves are presented as average percentages $\pm$ standard deviation (SD) from three independent experiments. Chi-square test was performed for analyses of embryos through development to the blastocyst stage. Yellow arrows indicate embryos exhibiting developmental arrest. * $p < 0.01$, bars = 50 μ m

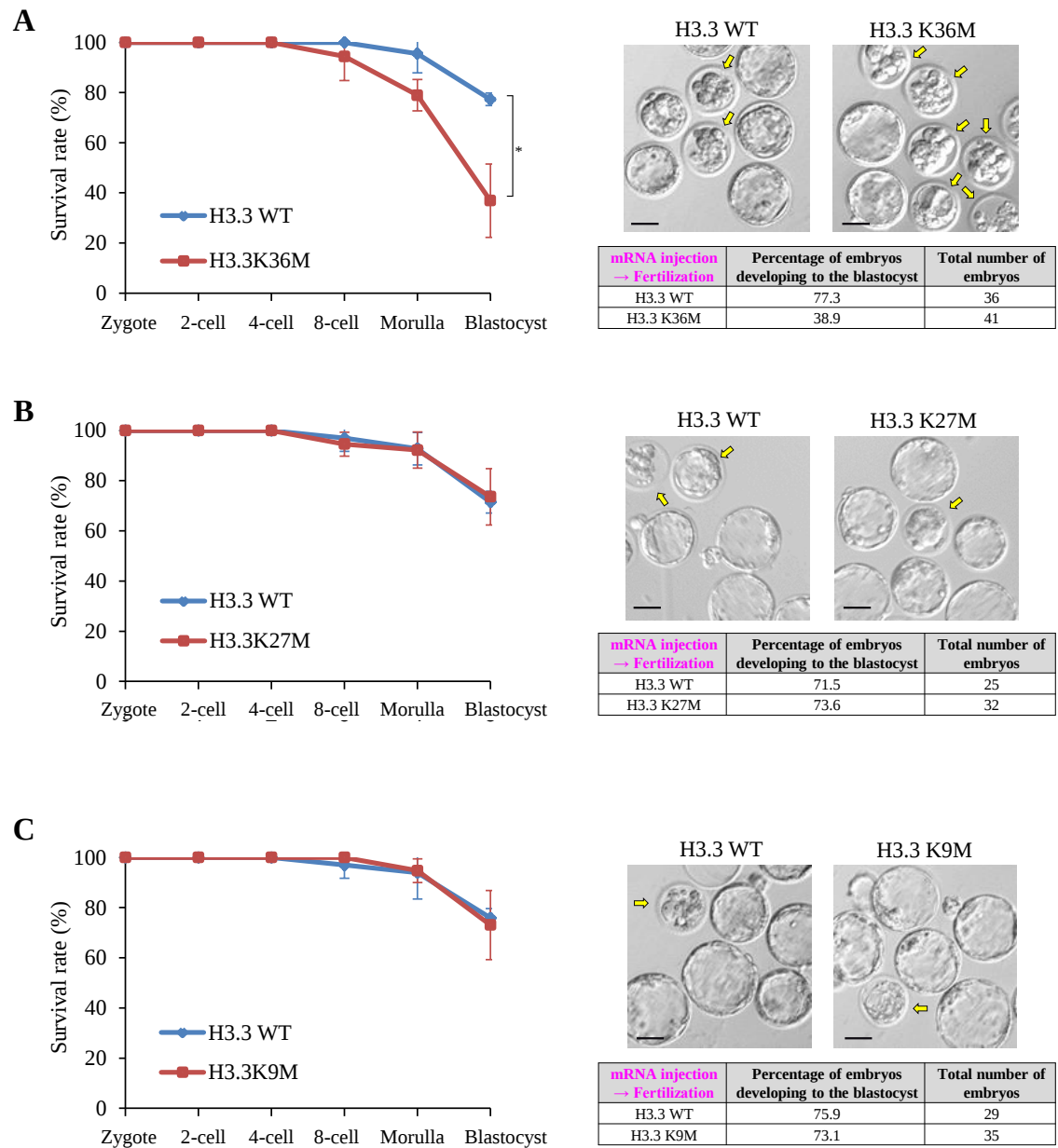


Fig. 4 H3K36 methylation is necessary for the early preimplantation development.

Developmental rate of zygotes overexpressed H3.3 K36M (A), H3.3 K27M (B) and H3.3 K9M (C), respectively, before fertilization. Each figure exhibited survival curves (Left), representative pictures (right top) and developmental rates (right bottom). Survival curves are presented as average percentages $\pm$ SD from three independent experiments. Chi-square test was performed for analyses of embryos through development to the blastocyst stage. Yellow arrows indicate embryos exhibiting developmental arrest. Bars = 50 μ m. * $p < 0.01$.

injected before fertilization (Fig. 4A), consistent with previous work demonstrating the importance of H3.3 K36 methylation for early embryonic development [31]. In contrast, K9M and K27M mutants did not exhibit any significant abnormalities (Fig. 4B, C). It was also found that the H3.3 K4R mutant, which itself is not methylated but does not affect endogenous H3K4 methylation, had no effect on embryonic development regardless of the order of mRNA injection and fertilization (Fig. 3B, C), implying that the inhibition of endogenous global H3K4 methylation is responsible for the phenotype.

To confirm the alteration of H3K4 methylation in H3.3 K4M-injected embryos, immunostaining in PN4–5 zygotes and 2-cell embryos were performed. The results clearly demonstrated a significant reduction of both H3K4me1 and H3K4me3 predominantly occurred in paternal PNs of PN4–5 zygotes, whereas the level of H3K4 methylation in maternal PN was relatively stable, although H3.3 K4M protein was comparably localized in both pronuclei. These results suggest that the effect of the H3.3 K4M mutant was dependent not only on the inhibition of SET domain-containing methyltransferases but also on chromatin incorporation, and that the maternal PN exhibited less dynamic H3K4me1 (Fig. 5) and H3K4me3 (Fig. 6). Furthermore, overexpression of H3.1 K4M did not alter endogenous K4 methylation even in paternal PN despite its nuclear localization, whereas it worked properly when it was overexpressed in 293T cells (Fig. 7), supporting the idea that the effect of the K4M mutants in zygotes is incorporation-dependent. At the 2-cell stage of H3.3 K4M-expressing cells, both H3K4me1 and H3K4me3 exhibited uneven nuclear localization, presumably because paternal PN-derived genomes are less methylated (Fig. 8 and 9). As expected, the H3.3 K4R mutant did not alter endogenous K4 methylation at either of the stages (Fig. 5, 6, 8 and 9), supporting the hypothesis that H3.3 K4M-induced growth retardation is likely due to the global reduction of H3K4 methylation.

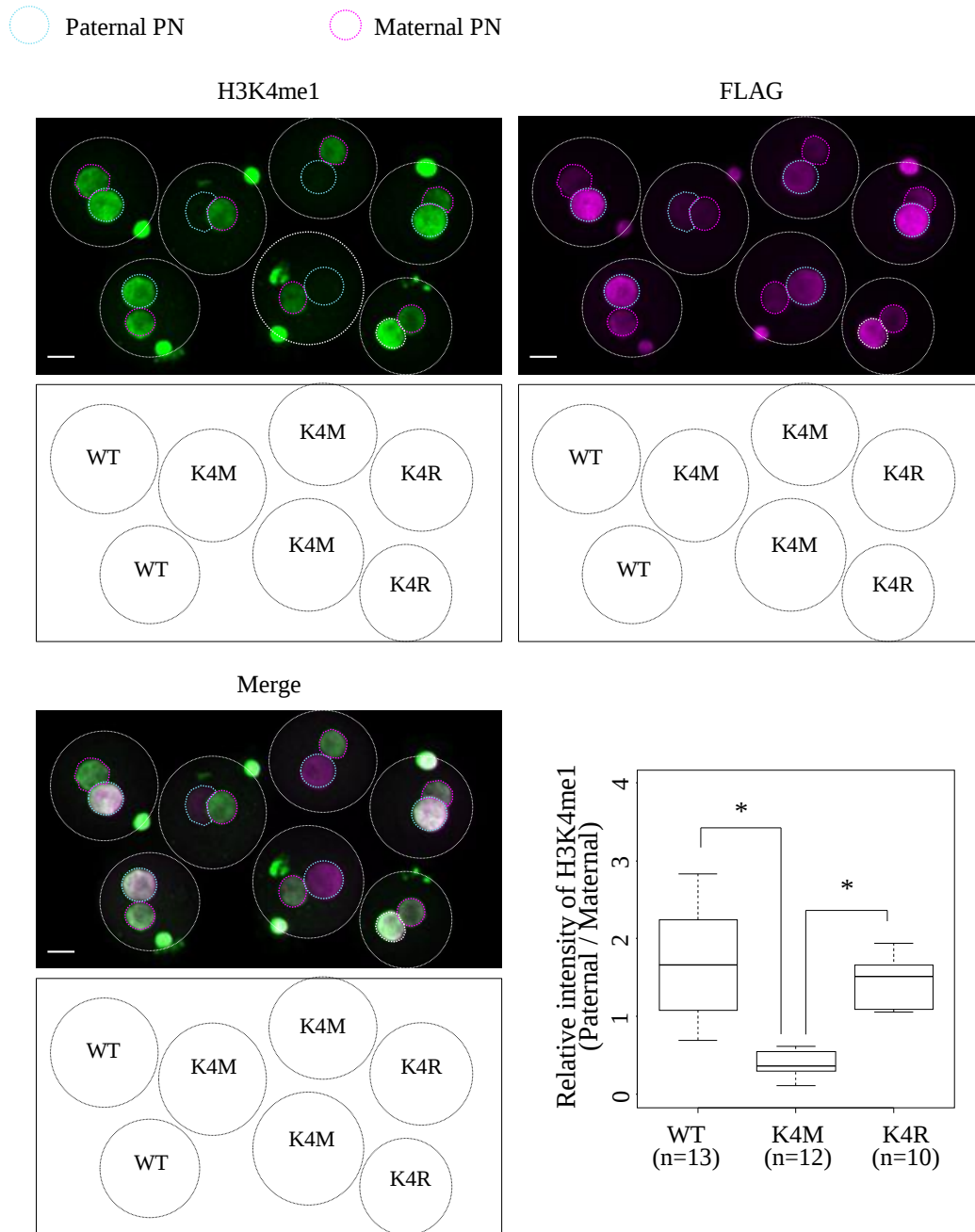


Fig. 5 Overexpression of H3.3 K4M causes paternal PN-specific decrease of global H3K4me1 in PN4-5 zygotes.

Immunostaining against H3K4me1 was performed for PN4-5 zygotes. White dashed lines indicated the edge of the PN4-5 PNs. Flag staining indicates the expression of H3.3 WT or H3.3 K4M mutants. Bars = 10 μ m. Each boxplot shows the quantified intensities of the attached images normalized by the intensities of paternal/maternal pronuclear intensity ratio. Sample numbers are as indicated. * $p < 0.01$,

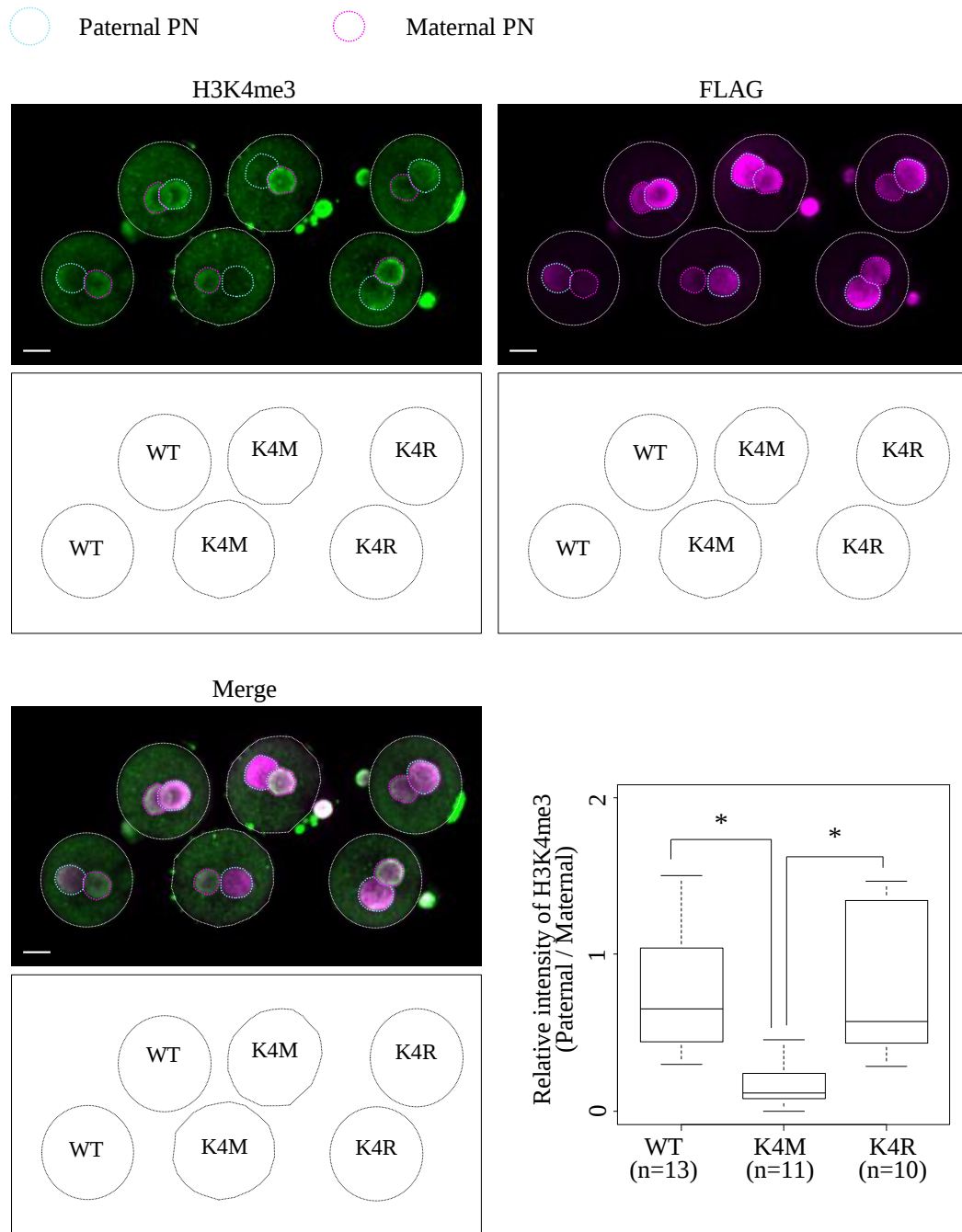
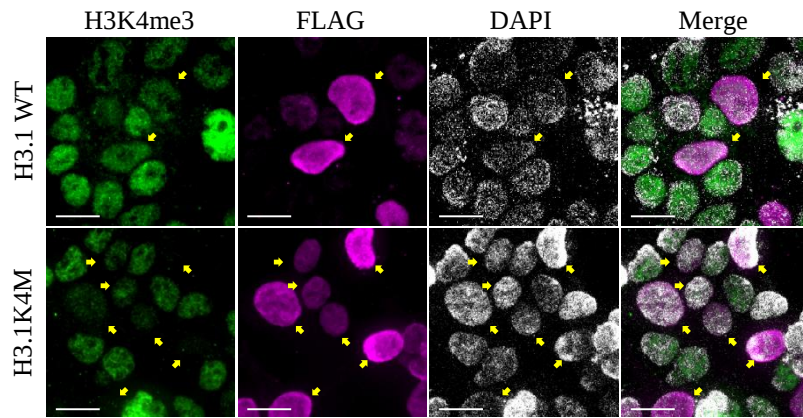


Fig. 6 Overexpression of H3.3K4M causes paternal PN-specific decrease of global H3K4me3 in PN4-5 zygotes.

Immunostaining against H3K4me3 was performed for PN4-5 zygotes. White dashed lines indicated the edge of the PN4-5 PNs. Flag staining indicates the expression of H3.3 WT or H3.3 K4M mutants. Bars = 10 μ m. Each boxplot shows the quantified intensities of the attached images normalized by the intensities of paternal/maternal pronuclear intensity ratio. Sample numbers are as indicated. * $p < 0.01$,

A



B

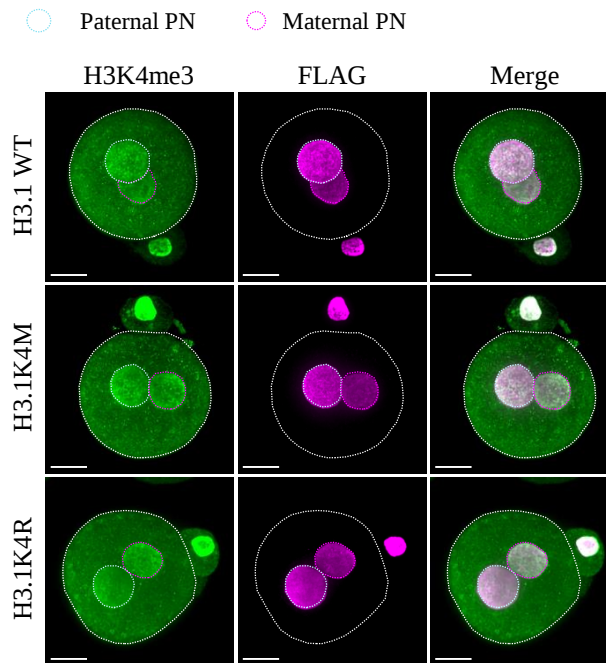


Fig.7 H3.1 K4M causes global decrease endogenous H3K4 methylation in 293T cells but not zygotes.

(A) Immunofluorescent images of 293T cells overexpressed H3.1 WT (upper panels) and H3.1 K4M (lower panels). Arrows indicated the cells in which H3.1WT or H3.1 K4M were overexpressed. Bars = 20 μ m. (B) Immunofluorescent images of PN4-5 zygotes overexpressed H3.1 WT (upper panels), H3.1 K4M (middle panels) or H3.1 K4R (lower panels), respectively. Bars = 20 μ m.

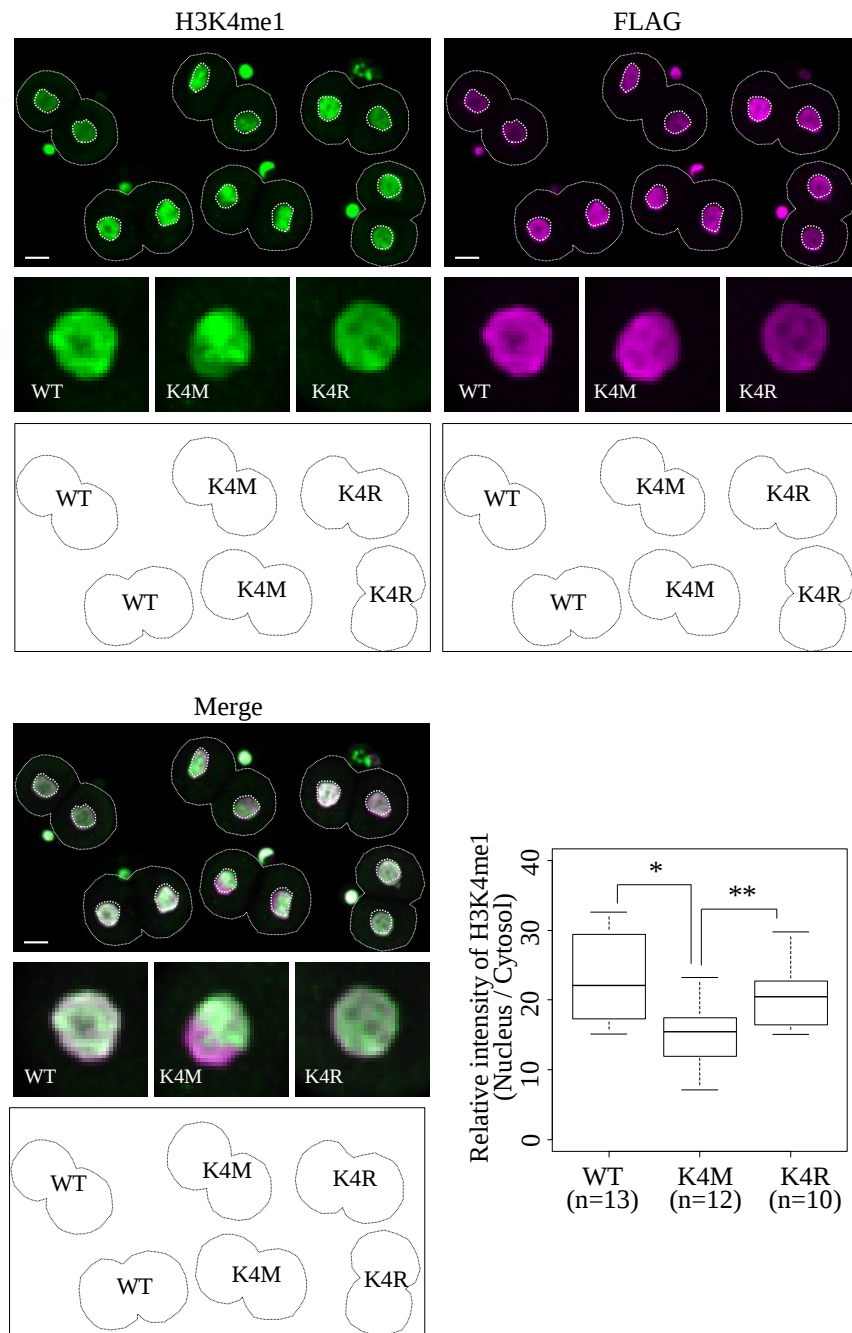


Fig. 8 Overexpression of H3.3K4M causes paternal PN-specific decrease of global H3K4me1 in 2-cell embryos.

Immunostaining against H3K4me1 was performed for 2-cell embryos. White dashed lines indicate the edge of the 2-cell cytoplasm. Flag staining indicates the expression of H3.3 WT or H3.3 K4M. Bars = 20 μ m. Each boxplot shows the quantified intensities of the attached images normalized by the paternal/maternal pronuclear intensity ratio. Sample numbers are as indicated. * $p < 0.01$, ** $p < 0.05$.

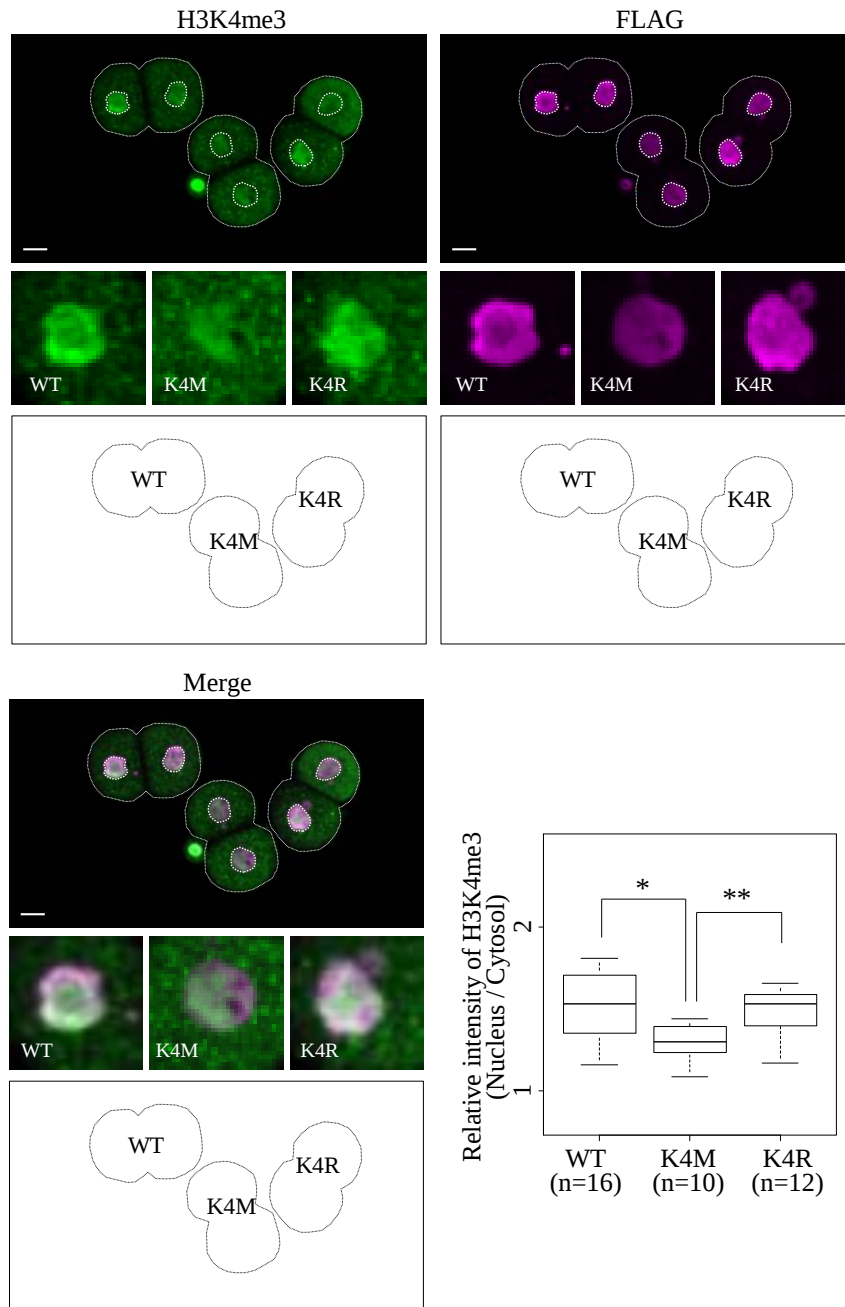


Fig. 9 Overexpression of H3.3K4M causes paternal PN-specific decrease of global H3K4me3 in 2-cell embryos.

Immunostaining against H3K4me3 was performed for 2-cell embryos. White dashed lines indicate the edge of the 2-cell cytoplasm. Flag staining indicates the expression of H3.3 WT or H3.3 K4M. Bars = 20 μ m. Each boxplot shows the quantified intensities of the attached images normalized by the paternal/maternal pronuclear intensity ratio. Sample numbers are as indicated. * $p < 0.01$, ** $p < 0.05$.

Minor ZGA in the paternal pronucleus is altered by the H3.3 K4M mutant

As H3K4 methylation is well known to be highly associated with active transcription, global transcription levels in H3.3 K4M-expressing embryos were examined using a 5-ethynyl uridine (EU) incorporation assay at the PN4–5 stages and the 2-cell stage, corresponding to the minor and major ZGA, respectively. Consistent with the levels of H3K4 methylation shown in Fig. 5 and 6, transcription was significantly altered in the paternal PN of H3.3 K4M-expressing zygotes, whereas H3.3 K4R had no such effect on transcription (Fig. 10). Although there was a modest decrease in transcription in H3.3 K4M-expressing cells at the 2-cell stage, no significant change in transcription was observed between samples (Fig. 11). Collectively, these data indicate that the growth retardation by H3.3 K4M mutants is caused by the alteration of minor, rather than major, ZGA in the paternal PN, and its onset is dependent on H3K4 methylation.

To further confirm the inhibitory effect of the H3.3 K4M mutant on paternal transcription during minor ZGA, the mRNA level of a paternally transcribed gene was compared between H3.3 wild-type (WT) versus H3.3 K4M injection conditions. For this particular experiment, a transgenic mouse line which carries a histone H4-Venus fusion transgene under the regulation of an endogenous histone H4 promoter was used (Fig. 12A) [60]. Weak but substantial expression of H4-Venus in zygotes and 2-cell embryos obtained from mating pairs of H4-Venus Tg sperm x WT oocytes were found (Fig. 12B). Comparison of the Venus mRNA levels between H3.3 WT- and H3.3 K4M-injected 1-cell embryos found a reduced level of Venus in H3.3 K4M-injected embryos (Fig. 12C). This result provided another piece of evidence that suppression of H3K4 methylation by H3.3 K4M attenuates paternal allele-derived minor ZGA.

Mll3/4 expression in the PN stage is necessary for early preimplantation development

There are six major histone methyltransferases that catalyse H3K4 methylation—*Setd1a*,

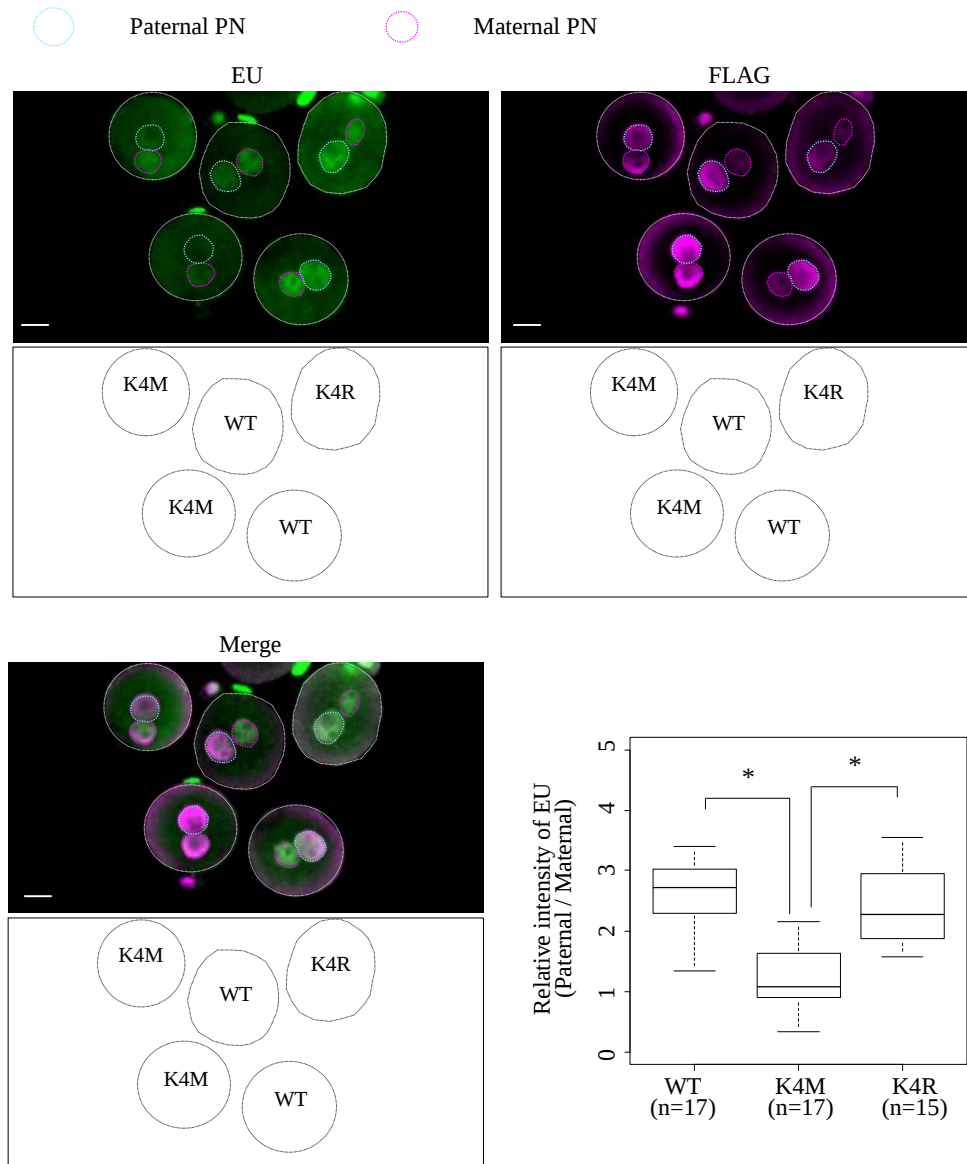


Fig. 10 Overexpression of H3.3K4M causes a reduction of global transcription predominantly in paternal PNs.

Representative fluorescent images of H3.3WT, H3.3 K4M and H3.3 K4R-overexpressed zygotes with EU treatment. Dashed lines indicate the edge of the PN4-5 PNs. Bars = 20 μm . Each boxplot shows the quantified EU intensities normalized by the paternal/maternal pronuclear intensity ratio.

* $p < 0.01$

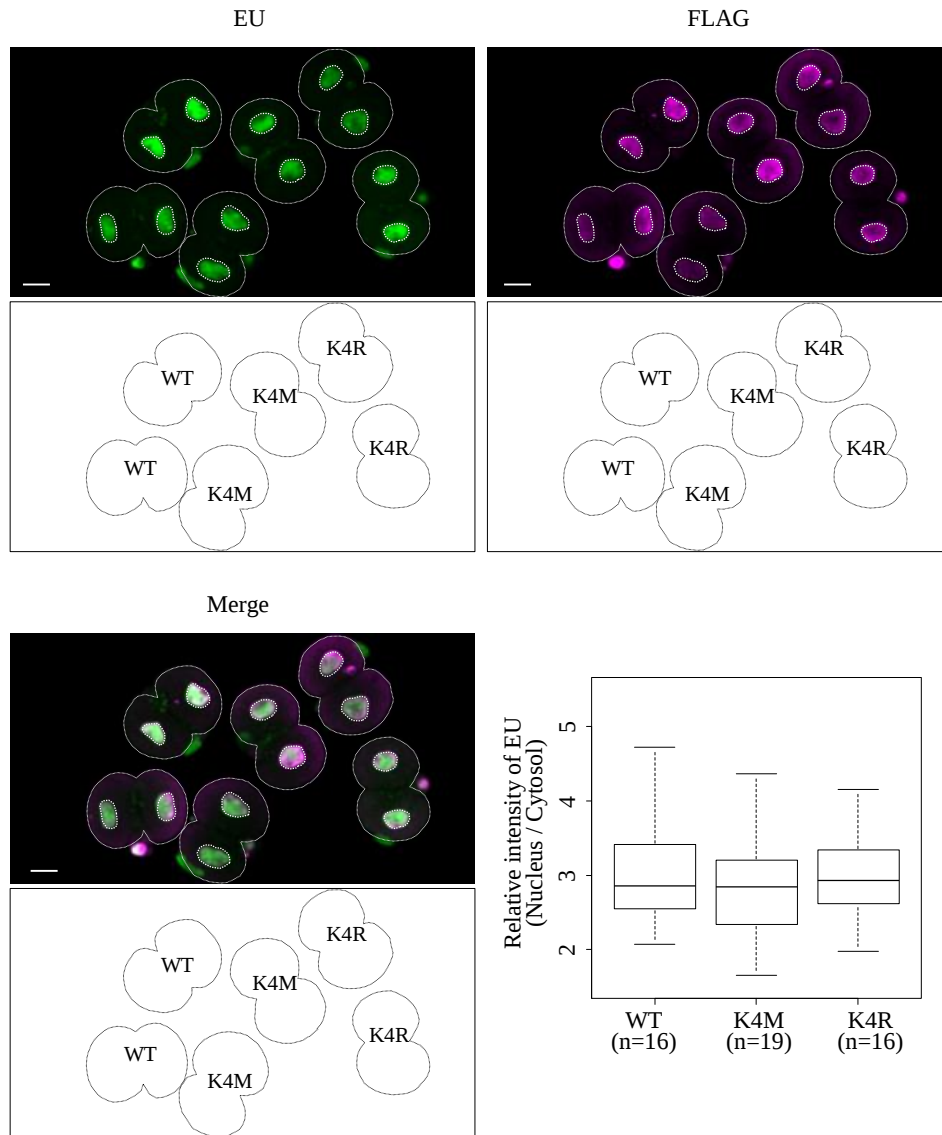


Fig. 11 Overexpression of H3.3K4M does not cause a reduction of global transcription predominantly in 2-cell embryos.

Representative fluorescent images of H3.3WT, H3.3 K4M and H3.3 K4R-overexpressed 2-cell embryos with EU treatment. Dashed lines indicate the edge of the cytoplasm. Bars = 20 μ m. Each boxplot shows the quantified EU intensities normalized by the paternal/maternal pronuclear intensity ratio. * $p < 0.01$

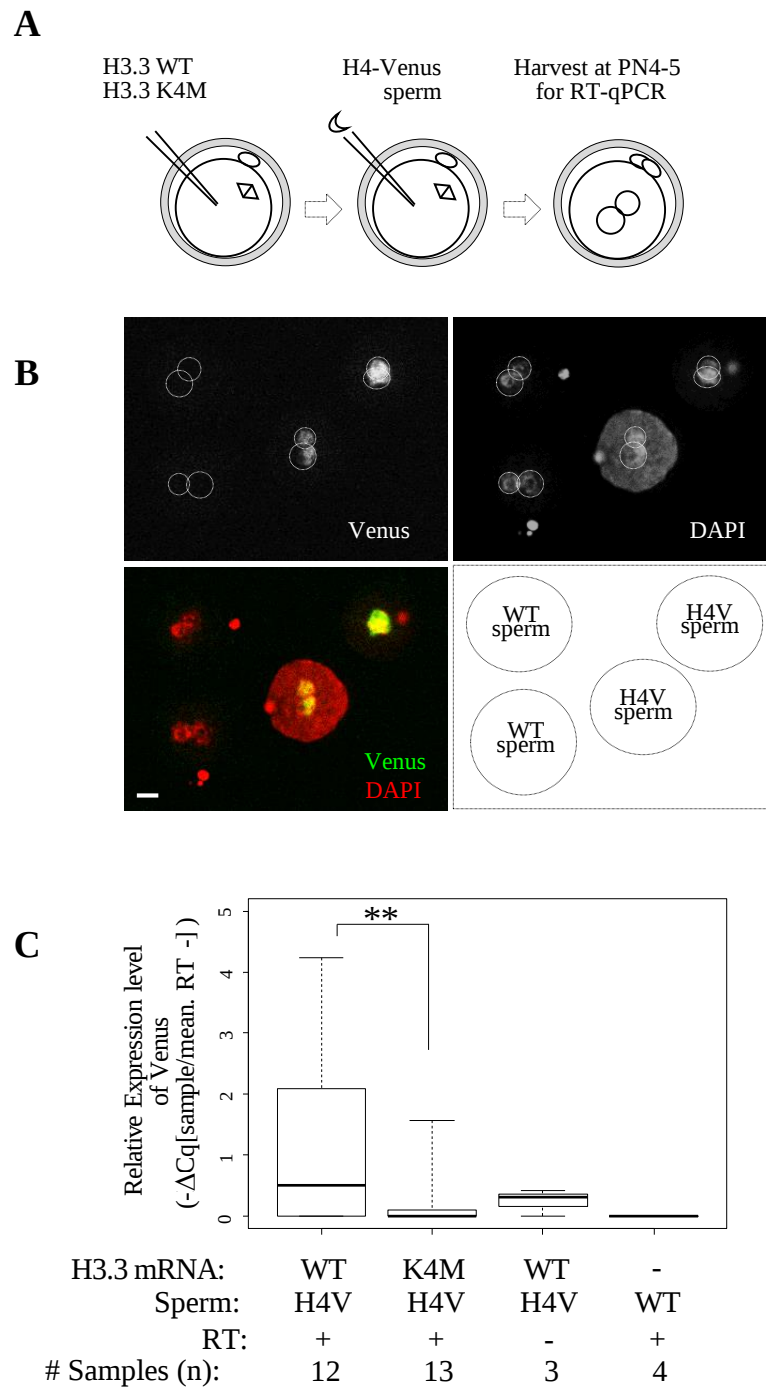


Fig. 12 Overexpression of H3.3K4M causes a reduction of H4-Venus expression derived from the paternal genome.

(A) Scheme of the experimental procedure for (B) and (C). (B) Representative image of sperm-derived H4-Venus expressing embryos. Bar = 20 μm . (C) Relative expression levels of H4-Venus by single cell RT-qPCR. Sample numbers are as indicated. ** $p < 0.05$

Setd1b, *Mll1*, *Mll2*, *Mll3*, and *Mll4*—which are divided into three distinct groups according to their sequence similarity and complex formation: *Set1a/b*, *Mll1/2* and *Mll3/4* [61-63]. To determine the enzyme responsible for H3K4 methylation during minor ZGA, their expression levels from MII oocytes to 2-cell stage embryos were first investigated using the Database of Transcriptome in Mouse Early Embryos (DBTMEE, <http://dbtmee.hgc.jp/index.php>) [64] [65]. The database indicated that among these H3K4 methyltransferases, *Mll3* showed transient expression highest at the 1-cell stage with a fragments per kilobase of transcript per million mapped fragments (FPKM) score of 81.5485, which was equivalent to that of *Actb* (also known as beta-actin; 90.2577) and higher than that of *Gapdh* (23.5705) (Table 2A). *Mll2* also displayed a high FPKM score (31.451), whereas *Mll4*, *Setd1a*, and *Setd1b* were expressed to much lower degrees, and *Mll1* was barely detectable (Table 2A). Interestingly, among these six enzymes, only *Mll3* and *Mll4*, both of which are orthologs of *Drosophila Trr* and have some functional redundancy, are categorized as “minor ZGA” depending on their expression patterns during the preimplantation stages (Table 2A). Because these six methyltransferases are composed of three distinct protein complexes (Table 2A) [66], the expression of molecules unique to each complex were further examined. The results showed that *Paxip1*, *Ncoa6*, and *Kdm6a*, unique components of the Mll3/4 complex, as well as factors that commonly exist in all three complexes (*Dpy30*, *Rbbp5*, *Wdr5*), were relatively abundantly expressed in preimplantation embryos and had a “minor ZGA” pattern of expression, supporting the hypothesis that *Mll3/4* is involved in H3K4 methylation in minor ZGA (Table 2B). The gene expression dynamics of the six methyltransferases according to pronuclear stage were verified by RT-qPCR, because the enzymes that function in minor ZGA (= the late pronuclear stages) could be transcribed in the early pronuclear stages so that there is enough time for them to be translated. Indeed, a transient increase in *Mll3* expression in the early pronuclear stages were found, suggesting that Mll3 is likely translated by the time of minor ZGA (Fig. 13). To further consolidate this idea, embryos were treated with

cycloheximide (CHX), an inhibitor of protein synthesis, at the time of fertilization. Interestingly, CHX treatment caused alteration of subnuclear localization and marked reduction of H3K4me1 and H3K4me3 in PN4-5, respectively (Fig. 14 and 15). Notably, retained H3K4me1 in CHX-treated embryos was accumulated in heterochromatic areas such as perinuclear and pericentromeric regions, where transcription was generally silenced (Fig. 16). Although this experiment did not specify Mll3/4 as responsible for zygotic H3K4 methylation, it supported the idea that *de novo* protein synthesis is required for full H3K4 methylation in minor ZGA.

Based on the gene expression pattern, the contribution of Mll3 and its paralog Mll4 in early preimplantation development were tested. *Mll3* and *Mll4* were knocked down by siRNA microinjection followed by ICSI using the same procedure as H3.3 K4M microinjection. To ensure that the siRNA was fully functional, siRNA microinjection into MII oocytes 8 hours before ICSI was performed in accordance with the protocol of a previous report [67], and confirmed that both *Mll3* and *Mll4* mRNA levels were decreased by ~85% and ~60%, respectively, at the 2-cell stage (Fig. 17A). Similar to the effects of overexpression of H3.3 K4M, single knockdown of *Mll3* caused developmental retardation at the 4-cell stage and arrest at the morula stage, whereas such alterations were not observed when siMll3 was microinjected after fertilization (Fig. 17B, C). On the contrary, single knockdown of *Mll4* did not significantly affect embryonic development, regardless of the order of siRNA injection and fertilization (Fig. 17B, C). However, simultaneous knockdown of *Mll3* and *Mll4* (siMll3&4) synergistically affected early embryonic development only when the knockdown was performed before fertilization (Fig. 17B, C), suggesting that the Mll3/4 is essential in early embryonic development.

A

Gene	Complex	RefSeq ID	Expression (FPKM)				Cluster	Correlation
			Oo	1C	2C	p1C		
<i>Setd1a</i>	Set1 complex	NM_178029	3.2596	3.0006	4.6672	4.5208	2-Cell Transient	0.9349
<i>Setd1b</i>		NM_001040398	2.5324	3.9732	1.2724	8.8600	-	-
<i>Mll1</i>	Mll1/2 complex	NM_001081049	0.0156	0.0604	0.7855	0.0735	2-Cell Transient	0.9983
<i>Mll2</i>		NM_001290573 NM_029274	27.0880	31.4509	9.7057	52.8020	Maternal RNA	0.9313
<i>Mll3</i>	Mll3/4 complex	NM_001081383	36.5327	81.5485	59.7321	98.3152	Minor ZGA	0.8229
<i>Mll4</i>		NM_001033276	3.8605	8.9460	5.3005	21.4913	Minor ZGA	0.9977
<i>Actb</i>	-	NM_007393	38.5702	90.2577	43.4170	156.4700	4-Cell Transient	0.9540
<i>Gapdh</i>	-	NM_001289726 NM_008084	8.9685	23.5705	9.2051	38.8086	1-Cell Transient	0.9711
<i>Rps18</i>	-	NM_011296	12.8851	3.4900	141.3360	4.0016	MGA	0.9943

B

Gene	Complex	RefSeq ID	Expression (FPKM)				Cluster	Correlation
			Oo	1C	2C	p1C		
<i>Wdr82</i>	Set1 complex	NM_029896	108.9050	142.5760	43.5376	146.0800	Maternal RNA	0.9512
<i>Men1</i>	Mll1/2 complex	NM_001168488 NM_001168489 NM_001168490 NM_008583	34.8539	19.3517	5.8675	9.0605	Maternal RNA	0.9441
		NM_008224	4.1618	5.1513	7.5938	8.0109	MGA	0.9801
<i>Hcfc1</i>	Mll3/4 complex	NM_018878	4.1535	5.4014	4.1693	7.8457	Minor ZGA	0.9192
<i>Paxip1</i>		NM_001242558 NM_019825	48.1207	87.6279	75.0216	115.8390	Minor ZGA	0.9858
<i>Ncoa6</i>		NM_009483	82.4269	138.6160	44.2918	127.8390	Minor ZGA	0.9979
<i>Kdm6a</i>								
<i>Dpy30</i>	Set1 complex	NM_001146222 NM_001146223 NM_001146224 NM_024428	155.4040	292.8240	175.3080	192.1310	Minor ZGA	0.9316
<i>Rbbp5</i>		NM_172517	29.3054	40.0043	12.4174	42.7093	Minor ZGA	0.9344
<i>Wdr5</i>	Mll3/4 complex	NM_080848	138.7120	263.3490	151.7520	292.1610	Minor ZGA	0.9939
<i>Ash2l</i>		NM_001080793 NM_001286207 NM_011791	21.0530	18.5563	18.3213	19.0263	Maternal to MGA	0.9512

Table 2 Gene expression patterns from DBTMEE

FPKM scores from RNA-sequence data for embryos at each stage. Oo = oocyte, 1C = 1-cell zygote, 2C = 2-cell embryo, p1C = parthenogenetic 1-cell. FPKM = fragments per kilobase of transcript per million mapped fragments.

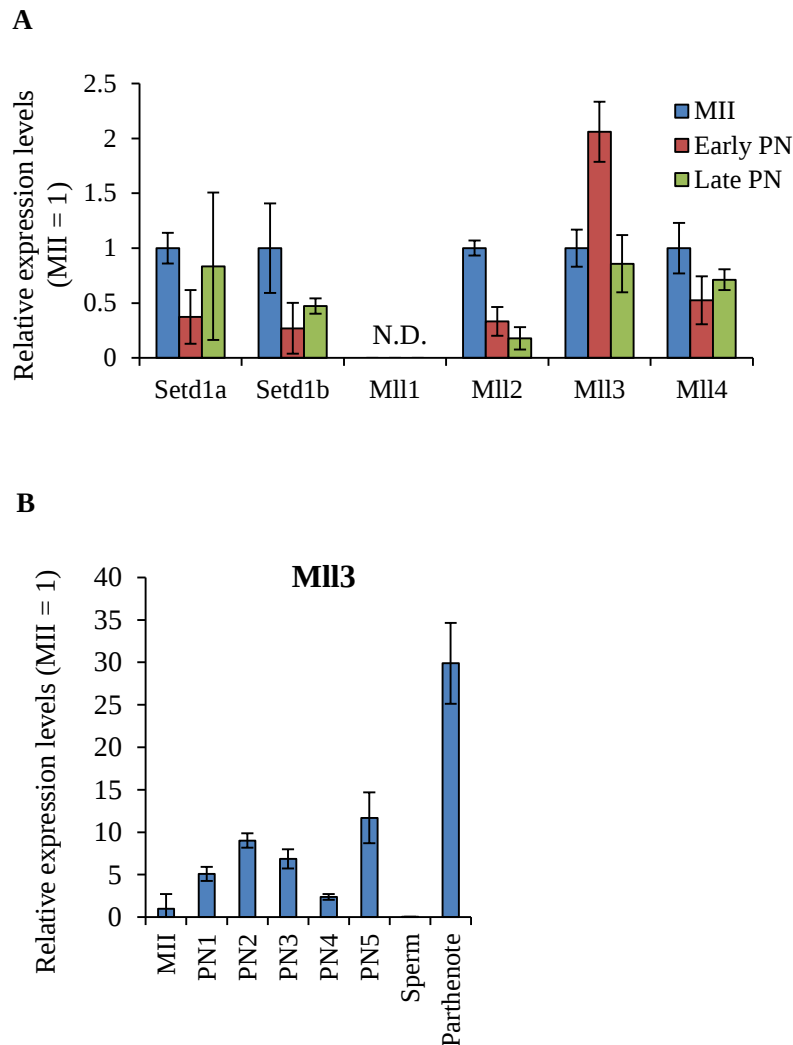


Fig. 13 Gene expression profiles of H3K4 methyltransferase

Relative mRNA expression levels of H3K4 methyltransferases were examined in MII oocyte, early PN (PN1-3) zygote and late PN (PN4-5) zygote (A), and relative mRNA levels of Mll3 was examined for oocyte, zygote, sperm and parthenote (B). Each data is presented as means $\pm$ SD from three independent experiments. The Y-axis indicates relative expression levels normalized with the geometric average of reference genes (*Ppia*, *H2afz*, *Hprt1*), and the value of MII set to 1 for each group.

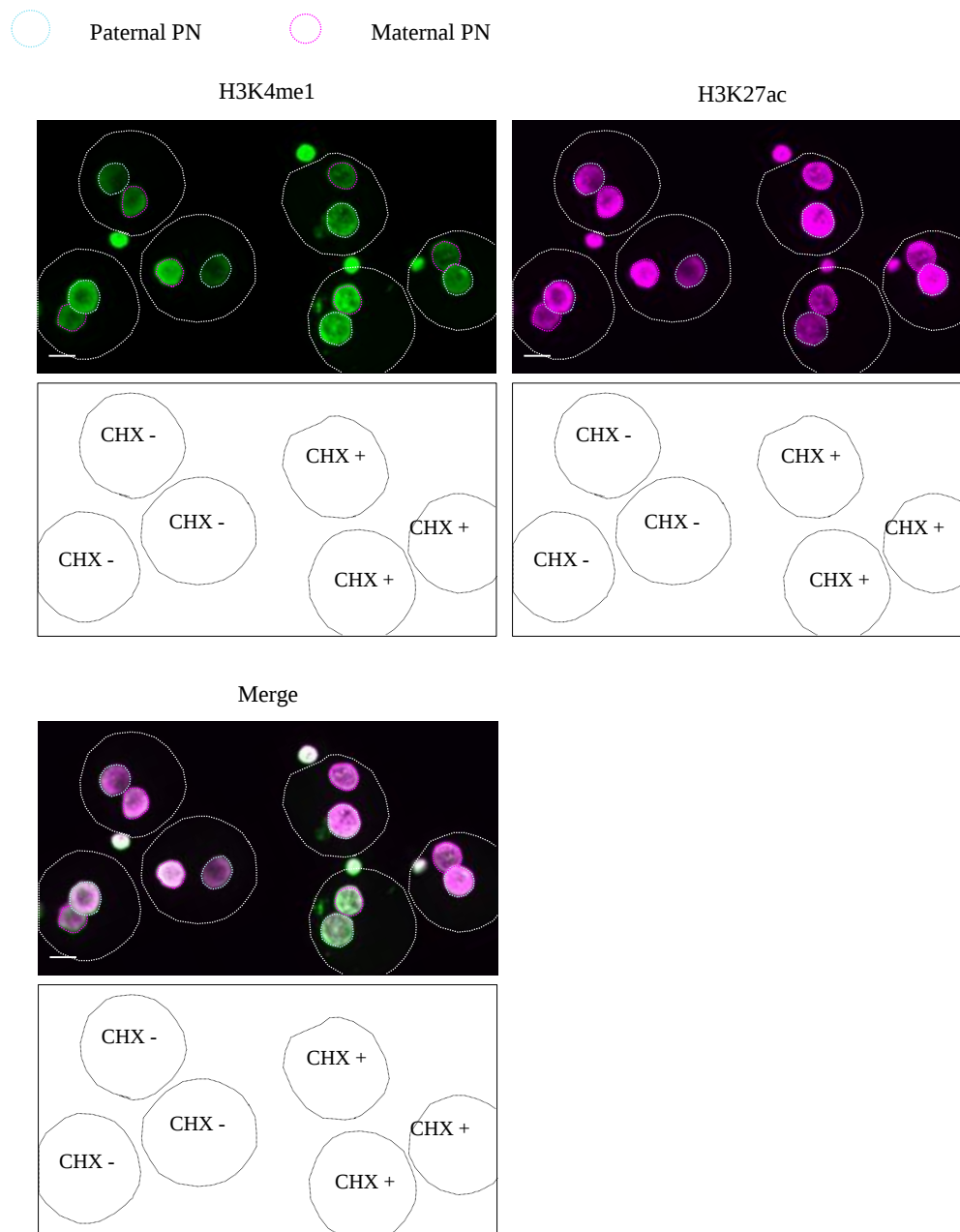


Fig. 14 Effect of CHX treatment on H3K4me1 in zygotes.

Effect of CHX on H3K4me1 in PN4-5 zygotes. Immunostaining of H3K27ac was provided as a nuclear marker. $n > 10$ for each experimental group. Bars = 20 μm .

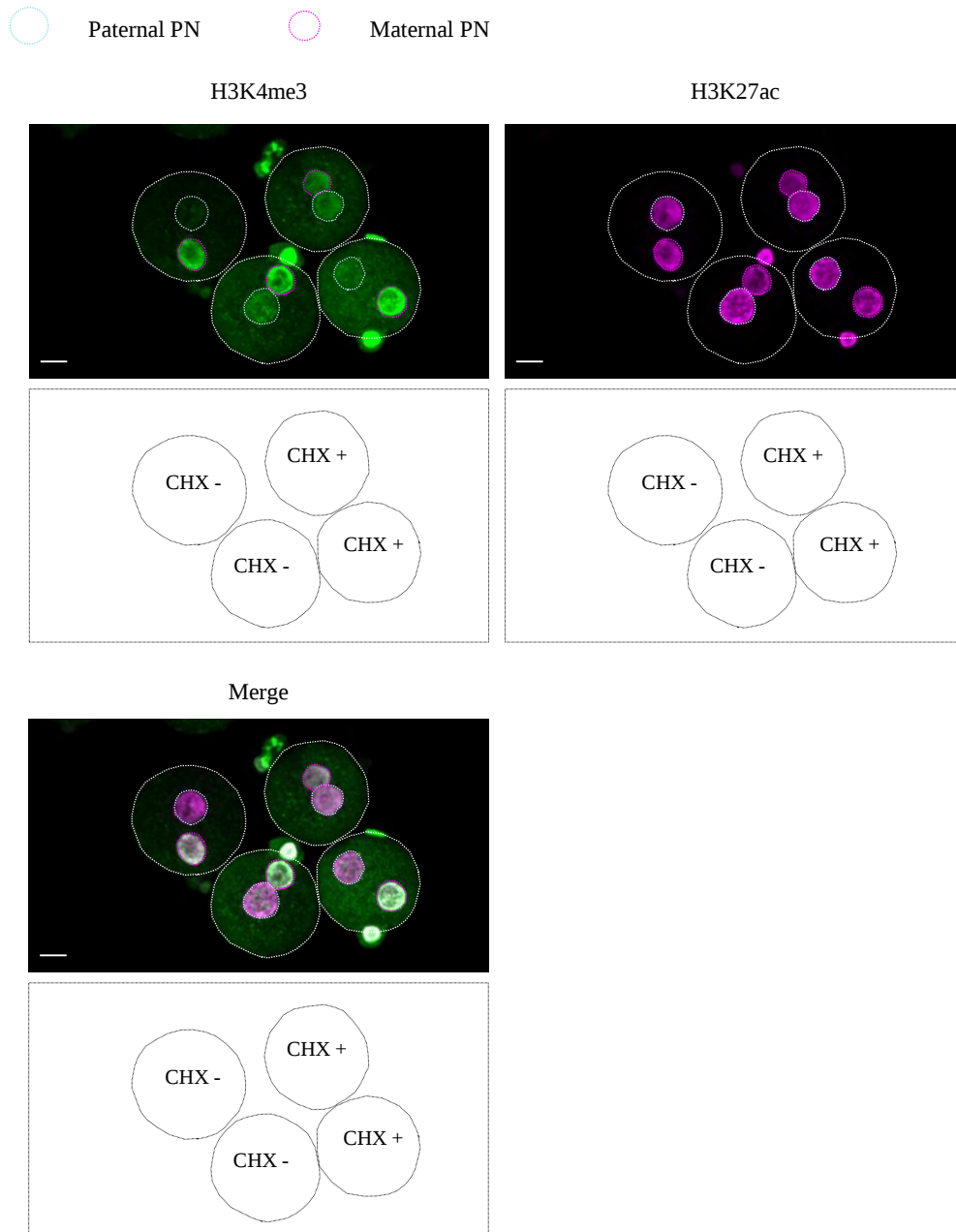


Fig. 15 Effect of CHX treatment on H3K4me3 in zygotes.

Effect of CHX on H3K4me3 in PN4-5 zygotes. Immunostaining of H3K27ac was provided as a nuclear marker. $n > 10$ for each experimental group. Bars = 20 μm .

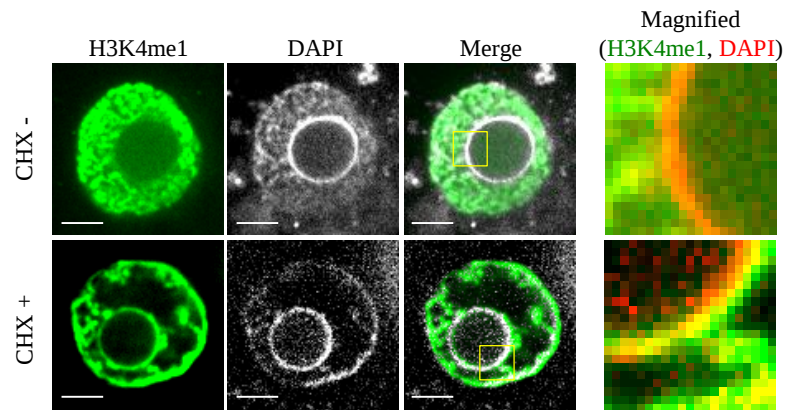


Fig. 16 Effect of CHX treatment on H3K4me1 localization in zygotes.

(left panels) Single slice Z-section image of paternal PN representing altered subnuclear localization of H3K4me1 by CHX treatment. Bars = 10 μ m. (right panels) Magnified images of areas indicated by yellow boxes in the merged images.

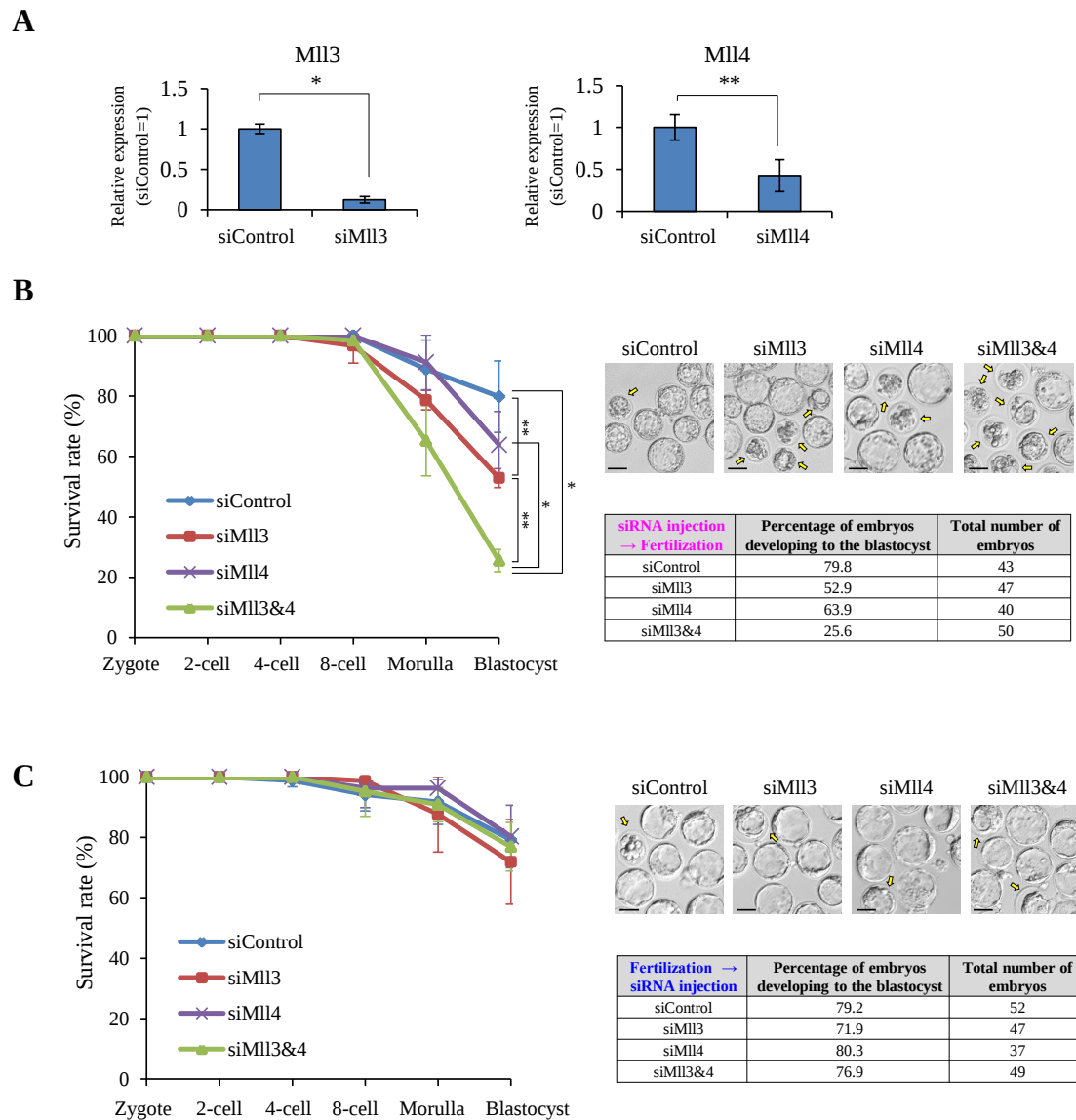


Fig. 17 Knockdown of *Mll3*&*4* before fertilization causes embryonic developmental arrest and *Mll4* exhibits a synergistic effect.

(A) mRNA expression of *Mll3* and *Mll4* were examined for 2-cell embryos which were microinjected with siMll3 and siMll4, respectively. Data are presented as means $\pm$ SD from three independent experiments. The Y-axis indicates relative expression levels with respect to the value of siControl-injected embryo, set to 1 for each group. (B-C) Developmental rate of zygotes with *Mll3* and/or *Mll4* knockdown before (B) and after (C) fertilization. Each figure shows survival curves (left), representative images at the blastocyst stages (right top), and developmental rates (right bottom). Yellow arrows indicate embryos exhibiting developmental arrest. * $p < 0.01$, ** $p < 0.05$, bars = 50 μ m

Recent studies have uncovered distinct functions for each Mll complex. The Mll3/4 complex is reported to be preferentially involved in enhancer activation through H3K4me1, in cooperation with Utx and p300, to remove H3K27me3 and ensure H3K27ac, respectively, rather than in promoter activation through H3K4me3. To examine whether siMll3/4 affected H3K4 methylation, immunostaining was performed in siMll3/4 zygotes followed by ICSI. However, no remarkable change in global H3K4me1 or H3K4me3 was observed in siMll3 or siMll3/4 embryos, respectively (Fig. 18). Nuclear accumulation of RNA polymerase II (RNAP II) C-terminal domain, including the non-phosphorylated form (Pan), the phosphorylated form at serine 5 representing transcriptional initiation (Ser5), and the phosphorylated form at serine 2 representing transcriptional elongation (Ser2), was also maintained in the knockdown embryos (Fig. 18). Similarly, Brg-1, a component of the switch/sucrose non-fermentable (SWI-SNF) chromatin remodelling complex, and previously reported to be involved in ZGA, was unaffected by siMll3/4 (Fig.18). These results suggested at least three possibilities. The first is that, since minor ZGA is a very weak transcriptional wave compared with major ZGA at the 2-cell stage, global changes in H3K4 methylation may not be especially correlated with transcriptional levels. The second is that Mll3/4 may simply not be responsible for global H3K4 methylation in zygotes. The third is that due to the abundant mRNA levels in oocytes, injecting siRNA into MII oocytes is too late to suppress their expressions (Table 2). To verify the second and third possibilities, siMll3/4 was injected into MI oocytes, followed by *in vitro* maturation for 30 hours and ICSI. As a result, a significant decrease of H3K4me1 in paternal PNs rather than in maternal PNs was observed in PN4-5 zygotes, confirming that Mll3/4 is responsible for paternal H3K4me1 in zygotes (Fig. 19 and 20A). Furthermore, reduction of H3K27ac was also induced by siMll3/4, implying a role of Mll3/4 in enhancer regions (Fig. 19 and 20B). Consistently, depletion of *p300*, the mRNA expression of which was significantly increased in PN1-3 stages, *via* siRNA injection also induced developmental arrest at around the 4-cell stage, also

indicating the importance of enhancers in minor ZGA (Fig. 21).

Finally, the effect of siMll3/4 on transcription was examined *via* an EU incorporation assay. A significant reduction in transcription in the paternal PN of *Mll3* and *Mll4* double-knockdown zygotes was observed (Fig. 22). Taking these results together, it is concluded that paternal H3K4me1 catalyzed by the Mll3/4 complex is required for minor ZGA in the paternal genome, and its alteration causes developmental arrest in preimplantation embryos.

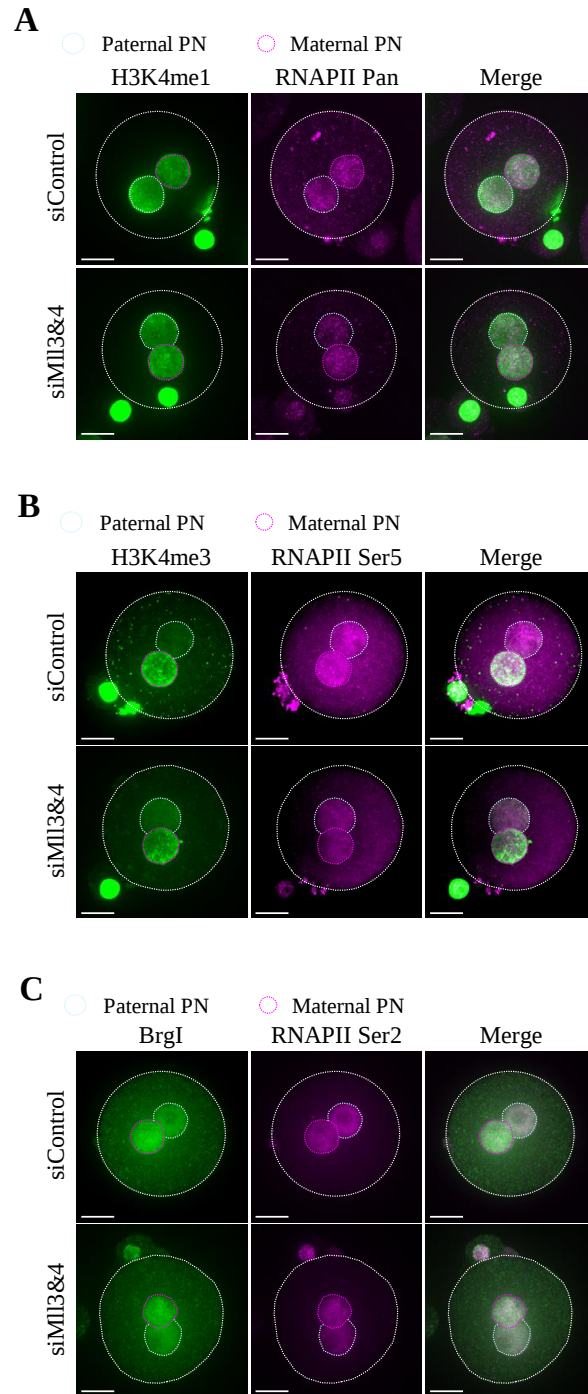


Fig. 18 Behavior and effect of transcriptional regulators involved in ZGA

Knockdown of *Mll3/4* does not alter the global H3K4me1/3 levels and the nuclear localization of RNAPII in zygotes. Immunostaining against H3K4me1 and PolII-pan (A), H3K4me3 and PolII-Ser5 (B) and Brg-1 and PolII-Ser2 (C) were performed in PN4-5 zygotes injected siControl or siMll3&4. Bars = 20 μ m.

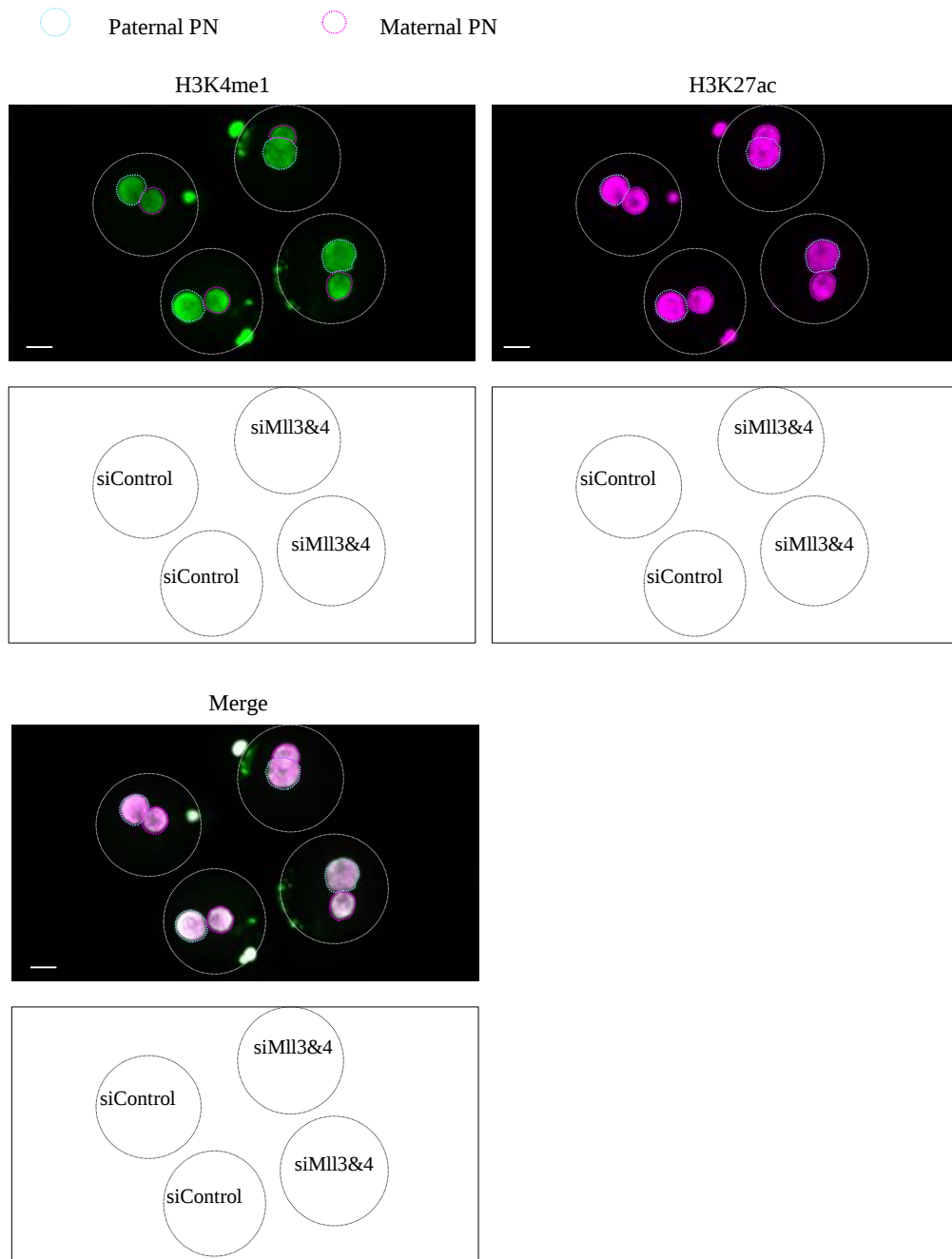


Fig. 19 Knockdown of *Mll3* and *Mll4* causes a reduction of global H3K4me1 and H3K27ac predominantly in paternal PNs.

Representative images of H3K4me1 and H3K27ac in zygotes microinjected with siControl or siMll3&4. Bars = 20 μm.

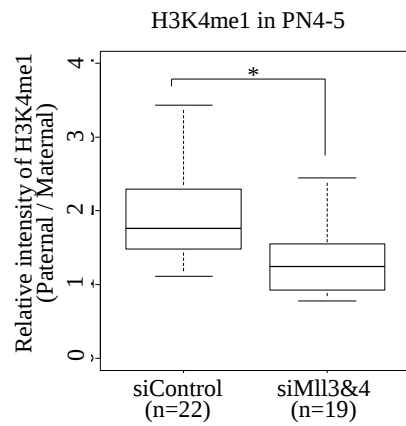
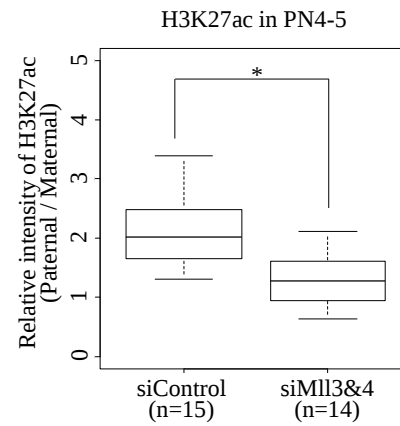
A**B**

Fig. 20 Quantitative analysis of global H3K4me1 and H3K27ac in *Mll3&4* knocked down zygotes.

Boxplots of quantified H3K4me1 (A) and H3K27ac (B) intensities. Sample numbers are as indicated. * $p < 0.01$.

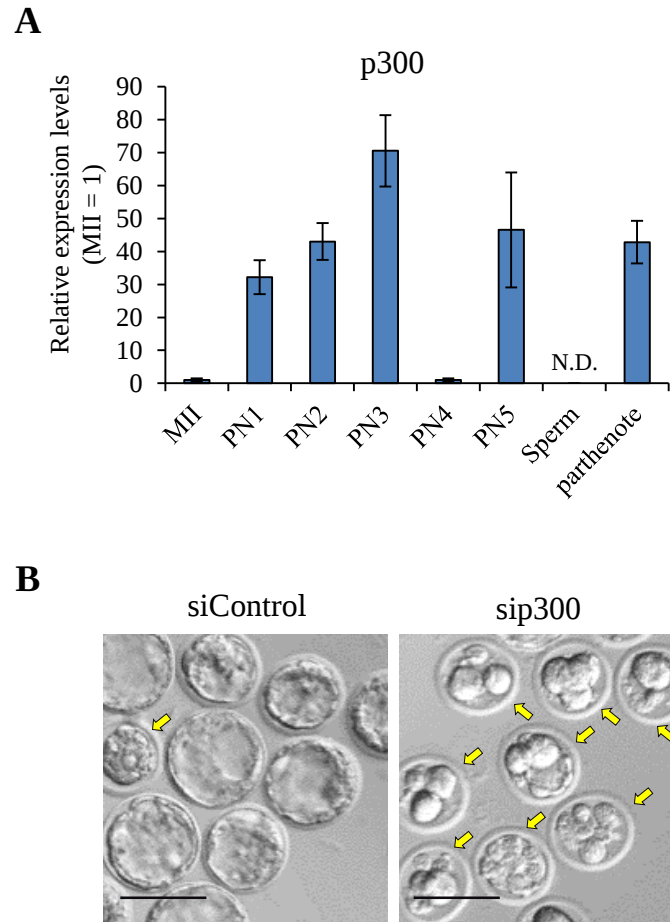


Fig. 21 Knockdown of p300 causes the developmental arrest of early preimplantation embryos. (A) Relative mRNA expression levels of *p300* were examined in oocyte, zygote (PN1-5), sperm and parthenote. Data are presented as means $\pm$ SD from three independent experiments. The Y-axis indicates relative expression levels, and the value of MII was set as 1 for each group. (B) Representative images of zygotes microinjected sip300 followed by ICSI at the blastocyst stages. Arrows indicate embryos exhibiting developmental arrest. Bars = 100 μ m

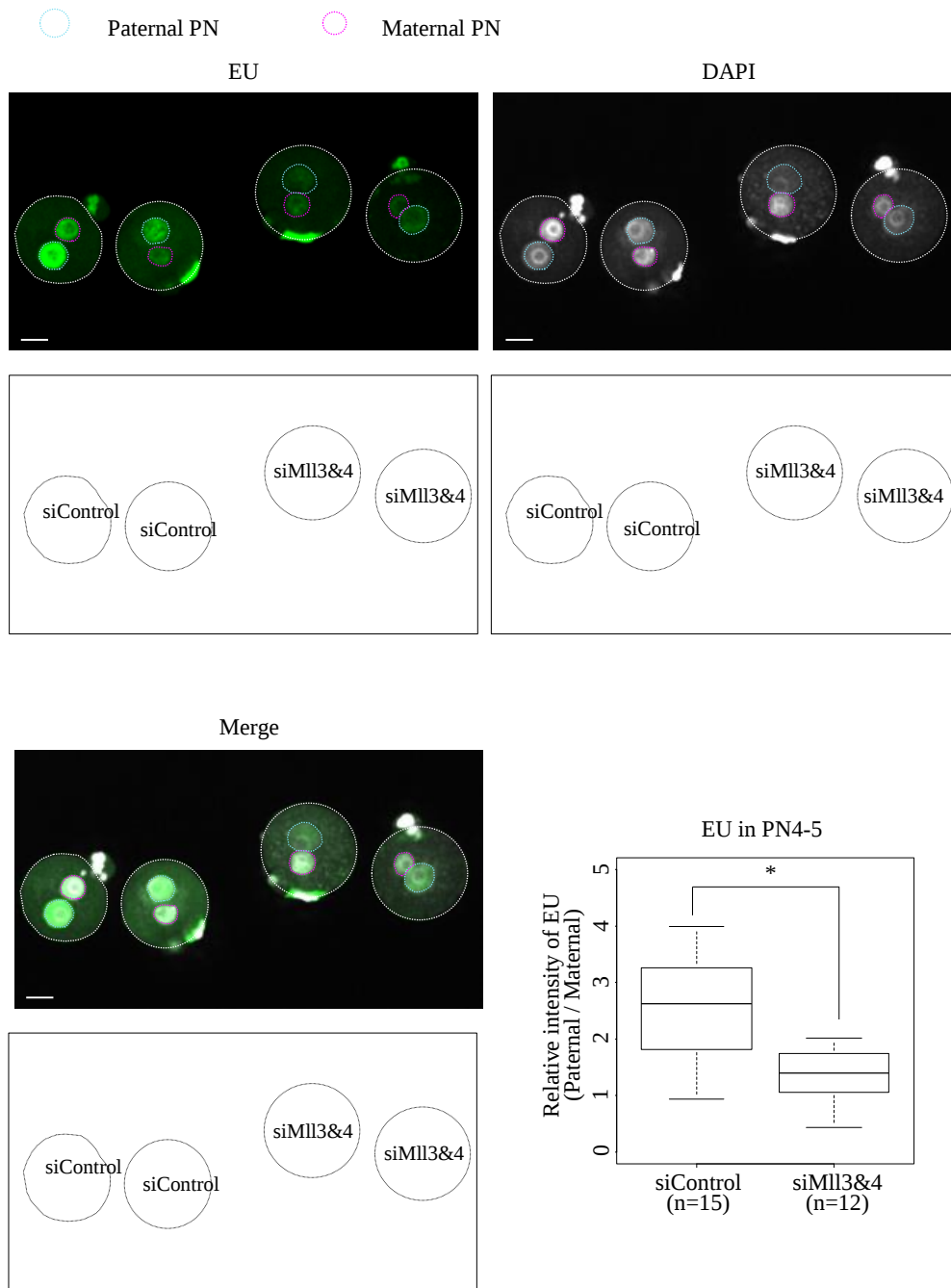


Fig. 22 Knockdown of *Mll3* and *Mll4* causes a reduction of global transcription predominantly in paternal PNs.

Representative images of EU staining in zygotes microinjected with siControl or siMll3&4. Bars = 20 μ m. Boxplots of quantified data from EU incorporation assay. * $p < 0.01$.

Discussion

Maternal to zygotic transition is a critical step for cells to convert from oocytes to embryos in terms of their genetic properties. This is thought to be a consequence of nuclear reprogramming, which induces the active transcription of zygotic genes, and plays pivotal roles in subsequent development. In mice, minor ZGA starts at the late pronuclear stages, which correspond to the first cell cycle of zygotes. It has been reported that the male PN exhibits higher transcriptional activity than the female PN due to its chromatin structure. After fertilization, protamines in sperm chromatin are rapidly removed and replaced by maternally expressed histones, which acquire various modifications as the zygotic stages proceed. During the zygotic period, some histone modifications in the paternal genome are quickly established and reach levels equivalent to those in the maternal genome, whereas others remain unmodified. Interestingly, Liu *et al.* recently reported that reprogramming factors are predominantly expressed from the paternal PN rather than the maternal PN [68]. However, it has been unclear whether and how this asymmetric epigenetic state influences transcriptional properties in minor ZGA.

In this study, K-M mutants to inhibit global histone methylation in zygotes were used, and it was discovered that H3K4 methylation is critical for the onset of minor ZGA in the paternal PN and subsequent preimplantation development. H3K4 can be mono (me1)-, di (me2)-, and tri (me3)-methylated, and it is closely associated with transcriptional activation. In maternal PNs, H3K4me1, -me2, and -me3 abundantly and stably exist throughout the zygotic stages, whereas in paternal PNs, H3K4me1 and H3K4me2 are established by PN2–3, while H3K4me3 begins to appear from PN4–5 [24, 26]. Although RNAP II and its associated phosphorylated forms are equally incorporated into the maternal and paternal PNs by the late pronuclear stages [69], several studies

suggest that the paternal PNs are more susceptible to transcription compared with the maternal PNs or the nuclei of the 2-cell stage [35, 36, 70], although the reason for this difference is still unknown. Consistent with these reports, transcriptional alteration by the H3.3 K4M mutant was also observed predominantly in a paternal PN.

Furthermore, *in silico* screening and siRNA analyses successfully identified that Mll3/4 derived from the maternal genome is likely responsible for the H3K4 methylation in paternal PNs. Previously, Mll2 was reported to be required for early embryogenesis, based on observations of ~30% reduction of major ZGA and developmental arrest mainly at the 2-cell stage when it was knocked out [71]. In Mll2-deficient zygotes, decreased H3K4me2 and H3K4me3 were observed in maternal PNs, whereas H3K4me1 was unaffected [71]. In contrast, Mll3 and Mll4 are key methyltransferases that establish H3K4me1 at enhancers rather than promoters [41, 45]. These observations that the injection of the H3.3 K4M prior to but not after fertilization is necessary to induce developmental defects strongly suggests that alteration of H3K4 methylation in the early PN stages is a cause of the abnormal phenotype (Fig. 3A). Also, considering that H3K4me1 in paternal PNs is already established in the early pronuclear stages, while the appearance of paternal H3K4me3 is not detected until the late pronuclear stages, the impairment of H3K4 methylation by H3.3 K4M mutant should mainly affect H3K4me1 rather than H3K4me3. Supporting this idea, knockdown of Mll3/4 in the early pronuclear stages also exhibited a similar phenotype, suggesting that H3K4me1 in paternal PNs occurs mainly at enhancers rather than promoters. Although further studies using, for example, Chromatin immunoprecipitation sequencing (ChIP-seq) to confirm that H3K4me1 at enhancers is required for the onset of minor ZGA, this study proposes a role for enhancers in the onset of minor ZGA based on data showing that knockdown of genes involved in enhancer activation, such as *Mll3/4* and *p300* in zygotes, consistently exhibited developmental alterations as

well as reduction in transcription in paternal PNs.

As the results have demonstrated here, K-M mutants are useful tools for examining the effect of SET domain-dependent histone methylation at specific residues by a forward genetic approach, especially when compared with the use of K-R mutants, which have been widely used previously to investigate alterations in histone methylation. In this study, it was confirmed that unlike the H3.3 K4M mutant, the H3.3 K4R mutants did not affect endogenous H3K4 methylation or early embryonic development, as previously reported by Santenard *et al.* [26]. Furthermore, developmental defects in the H3.3-K36M mutant seem consistent with a previous study demonstrating that exogenous expression of H3.3 WT, but not H3.3 K36R, could rescue developmental defects of preimplantation embryos treated with H3.3 morpholinos [31]. Unexpectedly, a data revealed that the H3.3 K27M mutant, in which paternal endogenous H3K27 trimethylation was markedly altered (Fig. 23), exhibits no significant effects in early embryonic development, conflicting with the findings of a previous study that demonstrated developmental alteration after injection of H3.3 K27R-EGFP mRNA. One possible explanation for this discrepancy is that there might be some differences in terms of chromatin incorporation efficiency between H3.3 K27M and H3.3 K27R, as K27M is reported to associate with a SET domain of histone methyltransferases to block their catalytic activity, possibly causing less incorporation of chromatin attenuating its effectiveness. Alternatively, the influence of an EGFP-tag, the size of which is relatively large compared with the size of H3.3, could be considered. Another possible limitation of the K-M mutants is that the phenotype may vary depending on the expression level. This conclusion is suggested by the failure to observe any changes of H3K4 methylation levels in the maternal genome after the 2-cell stage, presumably because the amount of H3.3 K4M protein was insufficient to replace endogenous H3.3 or to inhibit the H3K4 methyltransferases.

The role of several chromatin modifiers in ZGA has been reported previously. In addition to Mll2 [71], Brg-1 (also known as Smarca4), a catalytic subunit of the SWI/SNF chromatin remodelling complex, is highly expressed in zygotes, and loss of maternally derived Brg-1 in zygotes causes a ZGA phenotype including 2-cell stage arrest and reduced transcription of ~30% of the expressed genes, along with a reduction in H3K4me2 at the 2-cell stage [72]. Another example is Transcription intermediary factor 1 alpha (TIF1 α ; also known as Trim24): TIF1 α ablation in zygotes induces mislocalization of RNAP II, SNF2H, and Brg-1, and causes developmental arrest at the 2- and 4-cell stages [73]. Considering that α -amanitin, an RNA-synthesis inhibitor, blocks preimplantation development at the 2- to 4-cell stages, it is speculated that these factors are mainly involved in promoter H3K4 methylation during ZGA. In this case, however, significant changes in the localization of Brg-1 or RNAP II by siMll3/4 were not observed, and the growth arrest began only after the 4- to 8-cell stages. These differences may imply either a paternal-specific or an enhancer-specific effect of siMll3/4, or both. In addition, a previous genome-wide transcriptional study proposed a “stepwise” gene activation model that may be able to explain the delayed growth retardation. According to their model, one transcriptional wave triggers the following transcriptional waves during preimplantation development, and thus ZGA transcripts and their protein products would be required for the progression of embryos beyond the 4-cell stage [74].

In conclusion, this study not only demonstrates the importance of Mll3/4-mediated H3K4 methylation in the onset of minor ZGA in the paternal genome by utilizing a forward genetic and siRNA approaches, but also suggests the importance of enhancer activation for minor ZGA. These findings provide new insights into how the genome adapts during the maternal-zygotic transition: i.e., the transformation from a transcriptionally quiescent state at fertilization to the robust but not promiscuous gene activation thereafter. Further investigations into this topic may rely on innovative technical improvements such as a single-cell ChIP-seq.

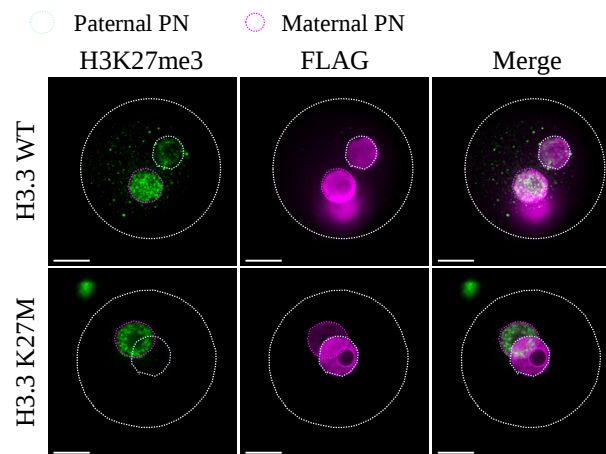


Fig. 23 Overexpression of H3.3K27M causes paternal PN-specific decrease of global H3K27me3 in PN4-5 zygotes.

Immunostaining against H3K27me3 was performed for PN4-5 zygotes. White dashed lines indicated the edge of the PN4-5 PNs. Flag staining indicates the expression of H3.3-WT or -K27M mutants. Bars = 20 μ m.

Summary

Epigenetic properties such as DNA methylation and histone modifications are dynamically altered predominantly in paternal pronuclei soon after fertilization. However, details concerning alterations in histone modifications remain elusive. To identify which histone modifications are required in early embryonic development, histone K-M mutants with single lysine (K) residues exchanged for methionine (M) were utilized, by which change the level of endogenous histone methylation at the mutated site is globally decreased. Four single K-M mutants for histone H3.3 K4, K9, K27, and K36, were prepared and the results successfully demonstrate that overexpression of H3.3 K4M in embryos before fertilization results in developmental arrest, whereas overexpression after fertilization does not affect development. Furthermore, loss of H3K4 methylation decreases the level of minor ZGA only in the paternal pronucleus. Furthermore, similar results from knockdown of *Mll3/4*, a H3K4 methyltransferase, were observed. Therefore, it was concluded that H3K4 methylation, likely established by *Mll3/4* at the early pronuclear stage, is essential for the onset of minor ZGA in the paternal pronucleus, which is necessary for subsequent preimplantation development in mice.

Chapter 2

Establishment of Alternative Culture Method for Spermatogonial Stem Cells Using Knockout Serum Replacement

Introduction

Recent reports have demonstrated that Spermatogonial stem cells (SSCs), the most primitive male germ cells in adult individuals, can be dedifferentiated into pluripotent stem cells under the specific culture conditions without any exogenous reprogramming factors such as Yamanaka factors [75-77]. Since there is no report that unipotent stem cells can be reprogrammed except SSCs, its detailed mechanism should be elucidated for further understanding of reprogramming. Experimental study on SSCs *in vitro*, however, is difficult because present culture methods strongly rely upon the quality of the BSA. Similar to the use of animal serum products, such as FBS, problems often arise because of inconsistencies due to variations between batches and manufacturers. Thus, the establishment of a completely defined culture medium for SSCs without animal products has been eagerly anticipated to promote the study on SSCs *in vitro*.

SSCs are responsible for the constitutive supply of sperm throughout life. Similar to other types of unipotent adult stem cells, SSCs undergo either self-renewal or asymmetric cell division, with the latter producing daughter cells (i.e., differentiated spermatogonia). The choice between self-renewal or differentiation is profoundly regulated by both intrinsic and extrinsic factors. The extrinsic factors are quite complex, because SSCs *in vivo* are surrounded by various types of somatic cells and differentiated spermatogonia. For example, sertoli cells exist in seminiferous tubules and support the growth of neighboring germ cells, both structurally and as a source of cytokines and hormones. Thus, it was difficult to identify the essential extrinsic factors for culturing SSCs *in vitro*, until Nagano *et al.* reported the first example of an *in vitro* culture method using feeder cells [78]. Subsequently, Kubota *et al.* generated chemically-defined culture conditions by the addition of 0.2% (w/v) BSA, and identified certain extrinsic cytokines, including glial cell line-derived neurotrophic factor (GDNF), GDNF family receptor alpha 1 (GFRA1) and fibroblast growth factor 2 (FGF2) [79,

80]. This improvement allowed SSCs to be cultured for longer periods without altering their undifferentiated properties, thus providing an unlimited supply of SSCs. Therefore, this method has been utilized for various SSCs studies, such as gene manipulation and molecular/biochemical experiments. Similarly, Kanatsu-Shinohara *et al.* also developed a distinct culture system for germ-line stem cells (GSCs) using StemPro34-SFM, a serum-free medium originally developed for human hematopoietic cell culture [81]. However, the culture of GSCs requires 1% (v/v) FBS as well as 0.5% (w/v) BSA and cytokines, including GDNF and FGF2 [81].

To establish defined culture method without animal products, the utility of Knockout Serum Replacement (KSR), a commercially available, defined cell culture supplement for culturing mouse SSCs was investigated, based on a recent report by Sato *et al.* [82]. In this report, KSR was successfully used for *in vitro* testicular organ culture without serum and cytokines. Interestingly, they also found that AlbuMAX, a lipid-rich, high-quality BSA, could be used as a substitute for KSR. These observations suggested that BSA could be replaced by KSR for culturing SSCs *in vitro*. The compatibility of KSR with SSCs isolated from various mouse strains, as well as the possibility of reducing the cytokine supplementation in the culture medium containing KSR were also examined.

Materials and Methods

Preparation of feeder cells

Mouse embryonic fibroblasts (MEFs) were isolated from ICR mouse embryos at embryonic day 13.5 (CLEA Japan). SIM mouse embryo-derived thioguanine- and ouabain-resistant fibroblasts (STOs) were purchased from the RIKEN BioResource Center Cell Bank (RCB0536). They were maintained in D-MEM (WAKO) supplemented with 10% FBS (EQUITECH-BIO, TX, USA), GlutaMAXI (Thermo Fisher Scientific) and penicillin/streptomycin (Thermo Fisher Scientific). After 3 or 4 passages, they were treated with 10 µg/ml mitomycin C (WAKO) for 2 hours. After the treatment, the cells were washed twice with PBS and trypsinized, and then 5.0 or 2.5×10^4 cells/well of MEFs and 7.5 or 3.75×10^4 cells/well of STOs were placed in 0.2% gelatin-coated 24 well- or 48 well-plates, respectively.

Culture medium

The composition of the basal culture medium for SSCs was described by Kubota and Brinster [80]. Briefly, MEM α was supplemented with GlutaMAX-I (Thermo Fisher Scientific), penicillin/streptomycin (Thermo Fisher Scientific), 2-mercaptoethanol (Thermo Fisher Scientific), 5 µg/ml insulin (Sigma-Aldrich), 10 µg/ml transferrin (Sigma-Aldrich), 0.5 ml/L Fatty Acid Supplement (Sigma-Aldrich), 7.6 ueq/L Free Fatty Acid mixture {0.4 µM Linolenic acid (Sigma-Aldrich), 1.0 µM Oleic acid (Sigma-Aldrich), 0.2 µM Palmitoleic acid (Sigma-Aldrich), 2.7 µM Linoleic acid (Sigma-Aldrich), 2.4 µM Palmitic acid (Sigma-Aldrich) and 0.9 µM Stearic acid (Sigma-Aldrich)}, 30 nM sodium selenite (Sigma-Aldrich), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Dojindo, Kumamoto, Japan), 60 µM putrescine (Sigma-Aldrich), 1 ng/ml FGF-Basic Human Recombinant (Peprotech, NJ, USA), 20

ng/ml Human GDNF Unconjugated (Peprotech) and 150 ng/ml Recombinant Rat GFR alpha-1 Fc Chimera CF (R&D Systems, MN, USA). MEM non-essential amino acids solution (Thermo Fisher Scientific) and MEM Vitamin solution (Thermo Fisher Scientific) were also added. Depending on the experimental design, 0.2% (w/v) BSA (MP Biochemicals, SA, USA) and 2 or 10% (v/v) Knockout Serum Replacement (Thermo Fisher Scientific) were added.

Isolation of SSCs

SSCs were isolated from postnatal day 8 testes of DBA/2NCrJCrJ (Charles River Laboratories Japan, Yokohama, Japan.), Jcl:ICR (CLEA Japan), C57BL/6JJcl (CLEA Japan) and 129X1/SvJJsSlc (Japan SLC, Shizuoka, Japan) mice, according to Kubota and Brinster [80]. Briefly, the testes were removed from the pups and washed with Hanks' Balanced Salt Solutions (HBSS) (Thermo Fisher Scientific), after removing the tunica albuginea. The testes were then digested by a solution of 5 mg/ml DNase I (Sigma-Aldrich) and 0.25% Trypsin-EDTA (Thermo Fisher Scientific), to disperse the seminiferous tubules. The reaction was stopped by adding FBS (Biofill, Victoria, Australia). The cells were filtered through a 35 µm pore-cell strainer (Corning, NY, USA) and were centrifuged (600 g, 4°C, 3 minutes). The pellets were resuspended in HBSS, and layered onto a Percoll solution, which was centrifuged without using the centrifuge brake (600 g, 4°C, 8 minutes). The pellet was washed with PBS supplemented with 1% FBS, 10 mM HEPES, 1 mg/ml glucose (Sigma-Aldrich), and penicillin/streptomycin (PBS-S), and was centrifuged again (600 g, 4°C, 3 minutes). The pellets were resuspended in PBS-S. Magnetic microbeads conjugated with anti-CD90.2 (Miltenyi Biotech, Gladbach, Germany) were added to the cell suspensions, which were incubated at 4 °C for 30 minutes. The cell suspensions were applied to an MS column (Miltenyi Biotech), and the magnetically retained cells were collected. After centrifugation at 600 g at 4 °C for 3 minutes, the pellets were resuspended with culture medium, and spread onto feeder

cells.

SSC transplantation

CD1-Foxn1nu (also known as ICR nude) male mice were used as recipients. 5-week-old recipients were treated with busulfan (Sigma-Aldrich, 40 mg/kg i.p.) to deplete endogenous germ cells. At 4–6 days after treatment, mice were subjected to bone marrow transplantation to avoid pancytopenia caused by busulfan treatment. Transplantation was performed at 5–6 weeks after busulfan treatment. SSCs were isolated from the P8 testes of DBA/2 mice carrying a histone H4-Venus gene, in which an H4-Venus fusion protein was expressed by a histone H4 promoter [60] as described above. Isolated SSCs were cultured for 3–4 weeks, and immediately before transplantation, SSCs were trypsinized and subjected to a feeder cell removal kit (Miltenyi Biotech) to eliminate MEFs. A total of 2.5×10^5 SSCs were injected per testis (n=4). At 8 weeks after transplantation, testes were removed from recipient mice for further examination.

Immunostaining of SSCs

After the culture medium was removed from the wells, the cells were washed with PBS, fixed with 4% paraformaldehyde (Wako) for 20 minutes at room temperature (RT), and then washed with PBS three times. Permeabilization was performed with 0.1% Triton X-100 at RT for 10 minutes, followed by two washes with PBS. Blocking was performed with 1.5% Blocking reagent (Roche, Basel, Switzerland) at RT for 1 hour. After blocking, the cells were incubated with the Anti-germ cell-specific antigen (GENA) monoclonal antibody clone TRA98 (Bio Academia, Osaka, Japan) (1:500) at 4°C overnight, and Alexa Fluor 488 goat anti-Rat IgG (Thermo Fisher Scientific) (1:1,000) was used for detection.

Feeder removal, RNA extraction and RT-qPCR

The SSCs and feeder cells were detached from the culture plates by trypsinization. The feeder cells were then removed by using Feeder Cell Removal microbeads (Miltenyi Biotech), according to the manufacturer's protocol. Total RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). The total RNA from mouse J1 embryonic stem cells (ESCs, ATCC SCRC-1010) was kindly provided by Dr. T. Kawamura, Kyoto University. Reverse transcription was performed by using the SuperScriptIII First Strand Synthesis System (Thermo Fisher Scientific), according to the manufacturer's protocol. qPCR samples were prepared with KAPA SYBR FAST qPCR Kit Master Mix (2X), using an ABI Prism Genetic Analyzer (KAPA Biosystems). The reactions were performed with ABI StepOne (Thermo Fisher Scientific). The PCR primers are listed in Table 3. The results were normalized to the expression level of ribosomal protein S7 (Rps7), because its expression level was stable and independent of the differentiation state of the SSCs (data not shown).

Cell cycle analysis by flow cytometry

The SSCs and feeder cells were detached from the culture plates by trypsinization and suspended in PBS-S. Cells were incubated with the Vybrant DyeCycle Orange Stain (Thermo Fisher Scientific) at 37°C for 30 minutes in the dark. They were filtrated by a 35 µm pore-cell strainer (Corning) and used for flow cytometry analysis by FACSCalibur flow cytometer (Becton Dickinson, NJ, USA). Cell cycle analysis was performed with Flowjo software (ver. 7.6.5. FLOWJO, OR, USA).

Statistical analysis

Student-*t* test was performed between two groups. Bartlett's test was used to check for

equality of variance, whereupon ANOVA and Tukey–HSD test were performed among more than two groups .

Ethics statement

All experimental procedures involving animals were approved by the Animal Experiment Ethics Committees at the Graduate School of Medicine, Kyoto University (MedKyo11094) and the Institute of Molecular and Cellular Biosciences, The University of Tokyo (#23015), respectively. The experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Kyoto University and the University of Tokyo. All efforts were made to minimize animal suffering and discomfort and to reduce the number of animals used.

Primer	Sequence	
	Forward (5'→3')	Reverse (5'→3')
Etv5	gatcagcaagtccttttatgg	aaatctgggacaaactgctcat
Gfra1	cagggaaatgacctactggaag	ttgttcctttggaaatgtgt
Ngn3	agactcccatacttcttggtga	aagacgcaacactggattaggt
Bcl6b	cgtgcttagcaatctgaatgag	tgaatagaagaagccactgcaa
Lhx1	agcgaaggatgaaacagctaag	gggaccgtagtactcactctgg
Kit	tgacaaattcacctcaaagtg	ggagttcacggatgtagacaca
Stra8	tggataagttgctgaagctcaa	gggactgtcctgaagaaaactg
Rps7	aaagccgtacgaaaaataagca	gtttcacacggatcctcttacc

Table 3 Primer sequences for RT-qPCR

Results

SSCs grow efficiently in the presence of KSR on MEFs

To examine the effect of KSR on the SSCs culture, four different combinations of BSA and KSR was tested as summarized in Fig. 24. In addition, the compatibility with two types of popular feeder cells (STOs and MEFs) was also examined (Fig. 24A). Among these conditions, 0.2% BSA and STOs (STO_BSA) corresponded to the conventional conditions reported by Kubota *et al.* [79]. After 6 weeks of culture on MEF feeder cells, a substantial cell growth was observed with 10% KSR alone (MEF_10K). The doubling time was 5.5 ± 2.7 days, which was equivalent to that of the conventional culture method (5.6 ± 0.2 days, [79]) (Fig. 24A). Remarkably, SSCs cultured with 0.2% BSA plus 2 or 10% KSR (MEF_B2K and MEF_B10K) exhibited the highest growth rates (the doubling times were 3.9 ± 0.2 days for MEF_B2K and 3.8 ± 0.1 days for MEF_B10K) as compared to the MEF_10K and other conditions (Fig. 24A, B). To the contrary, BSA alone (MEF_BSA) failed to support the cell growth, as most of the cells detached from the feeder cells before they formed colonies (Fig. 24A, B). These data suggested that KSR alone can maintain the *in vitro* growth of SSCs by substituting for BSA, when MEF cells were used as feeder cells. Furthermore, the addition of BSA to KSR significantly accelerated the cell growth, even though by itself it is incapable of supporting cell growth (Fig. 24A, B). In contrast, in the case of using STO feeder cells, only STO_BSA exhibited transient colony formation, which remained small, and eventually disappeared within 2 weeks (Fig. 24A, B). No proliferation of SSCs was observed in STO_10K, STO_B2K and STO_B10K after the culture was initiated, indicating that STO failed to support the growth of SSCs, even in the presence of both KSR and BSA (Fig. 24A, B).

Morphologically, MEF_B10K SSCs formed “grape-shaped” colonies, which were typical for SSCs colonies (Fig. 24B, inset). These colonies were all positive for GENA, a specific marker of

germ cells, whereas the numerous cell clumps observed in cultures with STOs contained very few GENA positive cells, indicating that they were composed of either dead germ cells or originated from the STOs (STO_BSA, STO_10K, and STO_B10K, Fig. 25).

Finally, whether SSCs cultured with KSR maintained the stemness *in vivo* was tested. Since previous studies revealed that the *in vitro* cultured cells usually consist of SSCs and non-stem cell progenitors, with the latter having lost their self-renewal capability [83], the MEF_BSA, MEF_10K and MEF_B10K SSCs were subject to transplantation into the testes of busulfan-treated ICR nude mice, in which their own germ cells were depleted. Unfortunately, however, MEF_BSA could not be tested, because a sufficient number of cells for the transplantation were not able to be obtained, due to poor cell growth (Fig. 24A). Unexpectedly, although MEF_B10K allowed the cells to proliferate significantly faster than MEF_10K *in vitro* (Fig. 24A), both MEF_10K and MEF_B10K cells were successfully engrafted with equivalent stem cell activity (Fig. 26A-C), strongly indicating that KSR alone is capable of maintaining the SSCs activity *in vivo*.

Gene expression properties are maintained in SSCs cultured with KSR

Since it had been confirmed that the SSCs cultured with KSR possess stemness *in vivo*, RT-qPCR was performed to investigate whether the gene expression properties of SSCs were influenced by KSR. Since KSR was originally developed to maintain embryonic stem cells (ESCs), the expression levels of *Nanog* and *Plzf* were first examined. *Nanog* is highly expressed in ESCs as well as primordial germ cells, but is disappeared in male germ cells by E16.5 [84], whereas *Plzf* is a marker of undifferentiated spermatogonia, including SSCs [85]. For this purpose, MEF_B10K was compared with STO_BSA, a previously reported conventional condition, and GSCs, which are also considered to be SSCs originated from gonocytes existing only in infant testis, and can be cultured *in vitro* in a distinct culture medium with a doubling time of approximately 2.6 days [81]. Mouse ESCs

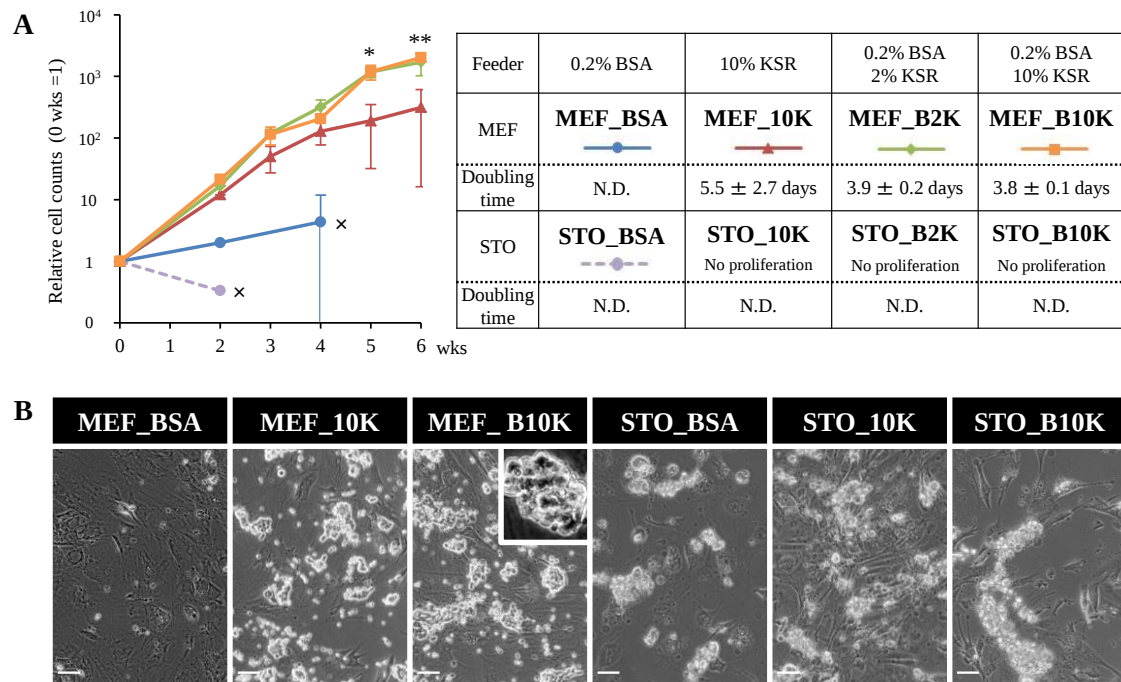


Fig. 24 KSR can substitute for BSA in SSCs cultures on MEFs

(A) Growth curves of SSCs cultured with the various combinations of BSA, KSR, MEFs and STOs (left panel) summarized in the table (right panel). Week zero indicates the day when the SSCs were isolated from postnatal day 8 testes of DBA/2 mice. The number of SSCs was counted at the indicated time points, and the cell counts are presented as means ± SD from three independent biological repeats. There are statistically significant differences in the data from 5 and 6 wks between MEF_B2K/B10K and MEF_10K (* $p < 0.01$, ** $p < 0.05$, ANOVA, Tukey's HSD test). Crosses (×) with STO_BSA and MEF_BSA indicate that cells could not be counted because of massive cell death and exfoliation. No proliferation was observed in SSCs cultured under the STO_10K, STO_B2K and STO B10K conditions. The calculated doubling times are also shown in the table (right panel). N.D., not determined due to inefficient proliferation. (B) Phase contrast images of SSCs from the indicated culture conditions. Scale bar, 100 μm. The magnified view shows a “grape-shaped” colony, which is typical for undifferentiated SSCs.

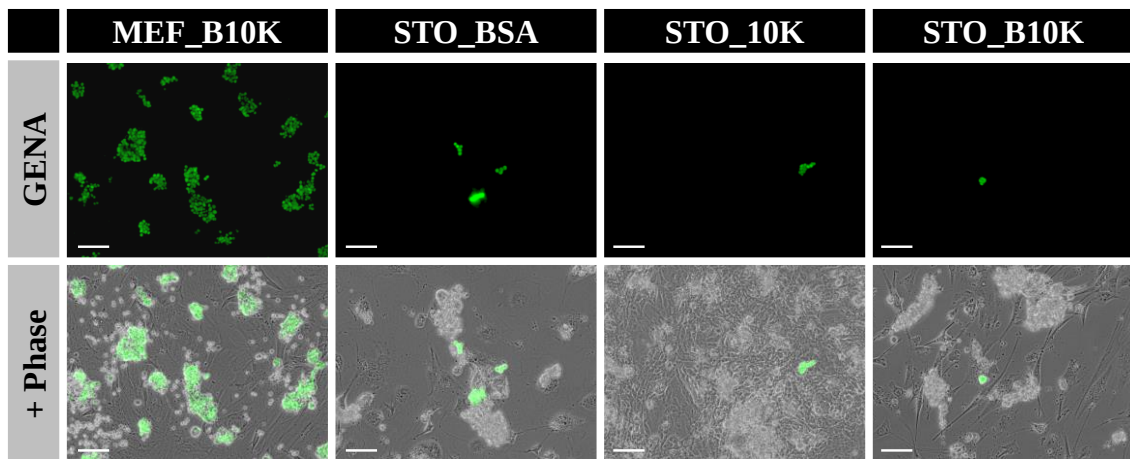


Fig. 25 “Grape-shaped” colonies in MEF_B10K are positive for GENA

Immunofluorescent analysis of SSCs, grown under different culture conditions, with the anti-GENA antibody, a germ cell-specific marker. GENA-positive cells are green in the upper panels, and phase contrast-merged images are shown in the lower panels. Scale bar, 100 μ m.

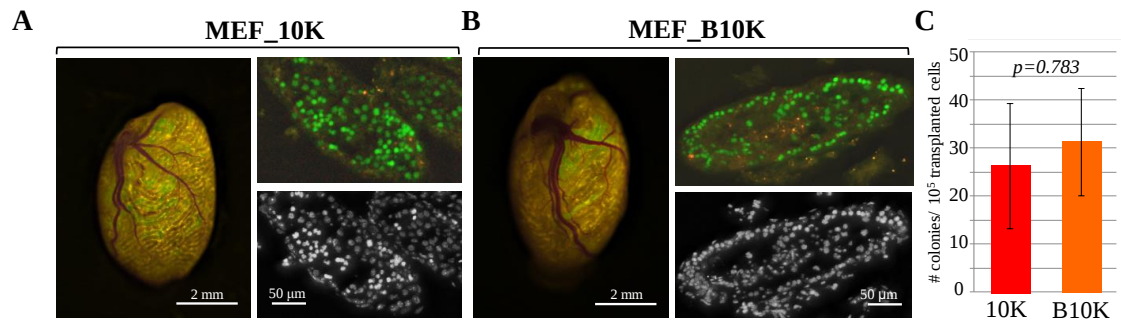


Fig. 26 SSCs cultured with KSR retain the stemness

(A, B) Representative images of mouse testes transplanted with Venus-expressed MEF_10K (A) or MEF_B10K SSCs (B), respectively. Gross Venus fluorescence images (left panels) and histological sections (Venus fluorescent images in upper panels, and Hoechst images in lower panels) are shown. Donor-derived Venus-positive cells are green, and the yellow fluorescence is caused by the auto-fluorescence of testicular tissue. Scale bars are as indicated. (C) Quantification of stem cell activities of MEF_10K and MEF_B10K by a transplantation assay. The graph presents the number of Venus-positive SSCs colonies per 10^5 transplanted cells $\pm$ SEM (n=3 per group). There was no significant difference between MEF_10K and MEF_B10K by Student- *t* test ($p=0.783$).

served as positive and negative controls for *Nanog* and *Plzf*, respectively. The results demonstrated that MEF_B10K produced higher levels of *Nanog*, as compared with STO_BSA, but it was comparable to GSCs (Fig. 27). The expression level of *Plzf* was similar among the three samples (Fig. 27), indicating that the cells cultured with MEF_B10K properly maintained the status of undifferentiated spermatogonia.

The mRNA expression levels in cells cultured on MEFs were compared with different combinations of KSR and BSA, as shown in Fig. 27, to see if the gene expression patterns were related to the growth rates. To evaluate the differentiation status, five markers for undifferentiated spermatogonia (*Gfra1*, *Etv5*, *Bcl6b*, *Lhx1*, and *Neurog3*) and two markers for differentiating/differentiated spermatogonia (*c-Kit* and *Stra8*) were tested. The cells in each group were prepared by changing the media from MEF_B10K to either MEF_BSA, MEF_10K, or MEF_B2K, and then culturing them for three weeks before isolating the RNA. However, the cells in the MEF_BSA culture started to detach from the feeder cells a few days after the medium was changed, as observed in Fig. 24A. Therefore, that culture was stopped at day 10, and isolated the RNA. As a result, all four samples cultured on MEF (i.e., MEF_BSA, MEF_10K, MEF_B2K and MEF_B10K) exhibited intermediate expression levels of undifferentiated spermatogonial markers, between those of GSCs and STO_BSA [75] (Fig. 28). However, no crucial differences in the expression levels of these genes were observed among the four samples, although the proliferation of MEF_BSA was quite limited. Similarly, the expression levels of *c-Kit* and *Stra8*, which are markers of differentiating/differentiated spermatogonia, were also unaltered, except for the subtle increase of *Stra8* in MEF_B10K (Fig. 28). Taken together, these results suggested that utilizing KSR is effective to ensure the cell proliferation *in vitro*, without altering the gene expression properties.

To further investigate how KSR facilitated the cell growth, the effect of KSR on the cell cycle was examined. At 5 days after changing the culture conditions from MEF_B10K to either

MEF_BSA or MEF_10K, a significant decrease of cells in G2/M phase in MEF_BSA was observed accompanied by a marked increase of cells in G1-phase and a subtle reduction of cells in S-phase (Fig. 29). MEF_10K exhibited the intermediate phenotype between MEF_B10K and MEF_BSA in each phase of cell cycle (Fig. 29), clearly suggesting that KSR can ensure the cell cycle progression, and the combined use of KSR with BSA further supports the effect on the cell cycle *in vitro*.

The addition of GDNF is sufficient in the presence of KSR.

The presence of certain cytokines is essential to maintain the stemness of SSCs *in vitro* by creating a favorable environment, and several cytokines and growth factors that enhance the proliferation of SSCs have been identified. Among them, GDNF, provided by the sertoli cells adjacent to the SSCs *in vivo*, is crucial for the self-renewal of SSCs *in vitro* as well [79]. In addition to GDNF, GFRA1 and FGF2 also support the continuous proliferation of SSCs *in vitro*. Since the combination of KSR and BSA (MEF_B2K or MEF_B10K) significantly facilitated the cell proliferation *in vitro*, it was next tested whether the presence of both KSR and BSA could allow the variety and/or amounts of cytokines to be reduced. Since MEF_B2K and MEF_B10K exhibited similar growth rates (Fig. 24), MEF_B2K was used as the basal medium, and four distinct culture conditions with reduced amounts/types of cytokines, as shown in Fig. 24, were tested to assess the effects on cell growth and gene expression.

The cell growth analysis revealed that the removal of GFRA1 and FGF2 did not affect the proliferation of SSCs as long as GDNF was supplemented, suggesting that these two cytokines were dispensable in the presence of KSR (Fig. 30). Furthermore, although the complete elimination of GDNF caused severe growth retardation, a 75% reduction in the GDNF concentration (from 20 ng/ml to 5 ng/ml) still supported continuous cell proliferation, with doubling times of 3.5 ± 0.1 days (20 ng/ml GDNF) and 4.0 ± 0.3 days (5 ng/ml GDNF) (Fig. 30A). The gene expression analysis

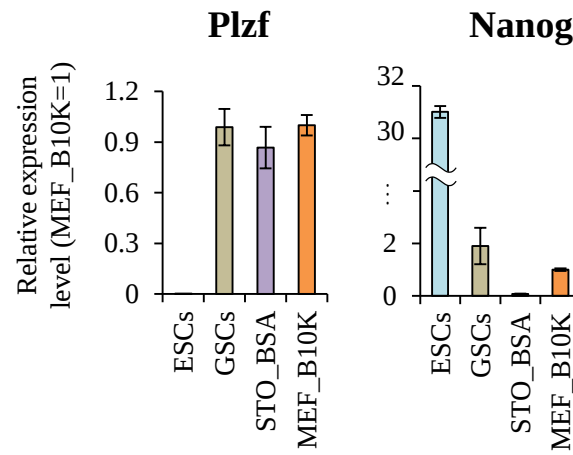


Fig. 27 Gene expressions for undifferentiated spermatogonia and ESC markers are maintained in SSCs cultured with KSR.

Relative expression levels of marker genes for undifferentiated spermatogonia (*Plzf*) and ESCs (*Nanog*). Data are presented as means $\pm$ SD from three independent experiments. The Y-axis indicates the relative expression level, with the value of MEF_B10K set to 1 for each group.

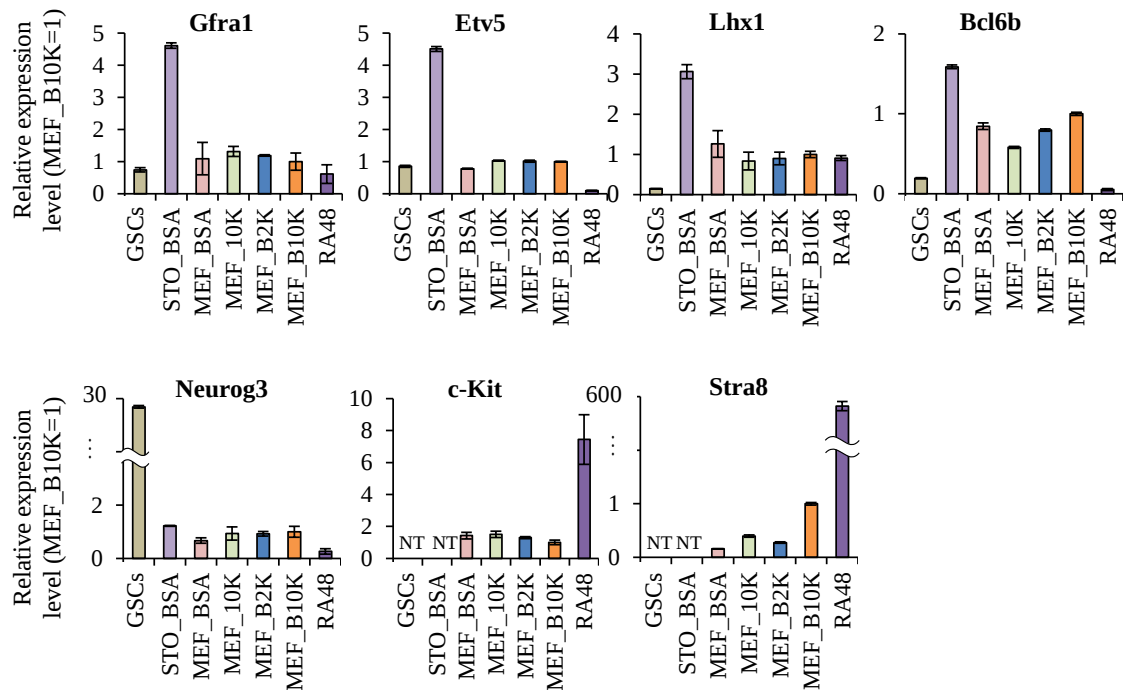


Fig. 28 Gene expressions for undifferentiated and differentiated spermatogonial markers.

Relative expression levels of other undifferentiated spermatogonial markers (*Gfra1*, *Etv5*, *Lhx1*, *Bcl6b*, and *Neurog3*) and differentiated spermatogonial markers (*c-Kit* and *Stra8*). Data are presented as means $\pm$ SD from three independent experiments. The Y-axis indicates the relative expression level, with the value of MEF_B10K set to 1 for each group.

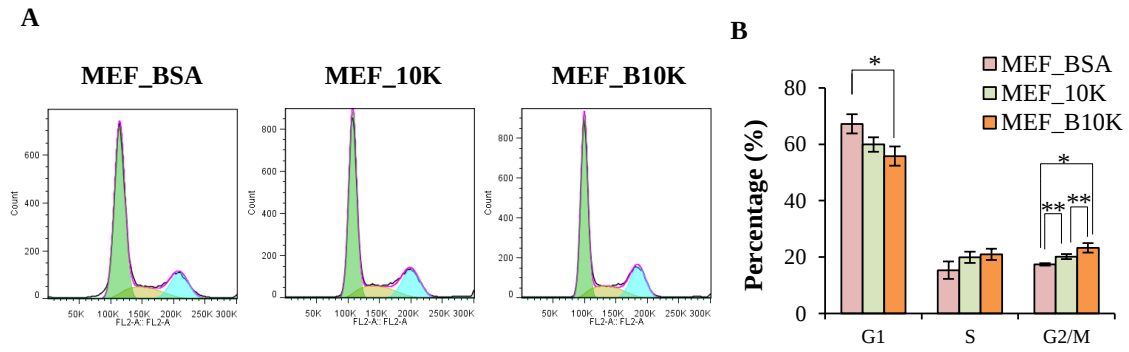


Fig. 29 Cell cycle analysis of SSCs cultured with KSR

(A) Cell cycle analyses by flow cytometry at 5 days after the medium change from MEF_B10K to each indicated condition. Histograms are representative examples of each sample. The tables under each histogram indicate the percentages of each cell cycle stage. The histograms are colored according to each cell cycle stage. Green: G1 phase. Ocher: S phase. Light blue: G2/M phase. (B) Quantification of the results shown in (A). The graph is presented as means of the percentages $\pm$ SD from three independent experiments. Asterisks indicate statistical significance (* $p < 0.01$, ** $p < 0.05$, ANOVA, Tukey's HSD test).

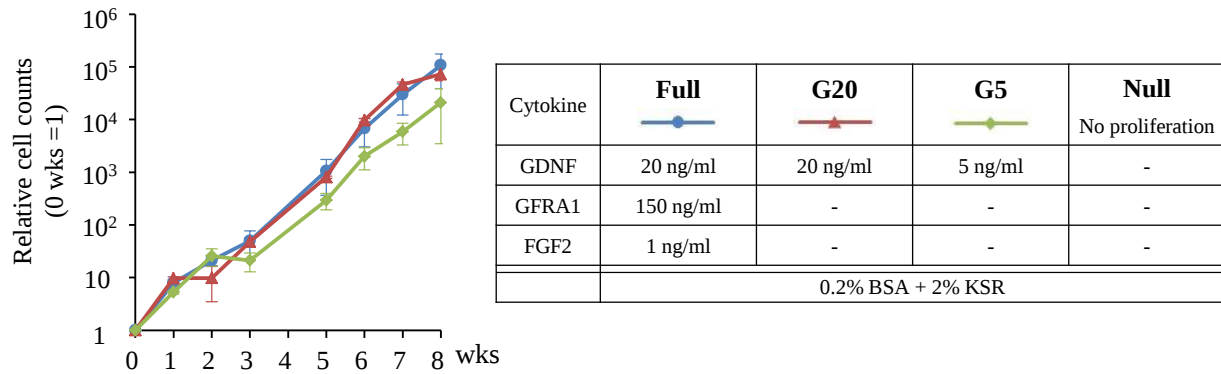
revealed that the expression levels of undifferentiated markers (*Etv5*, *Gfra1*) were maintained even when the amounts of the cytokines were reduced (Fig. 30B). On the other hand, the expression levels of *Neurog3* and *c-Kit* were greatly increased without GFRA1 and FGF2, but the cell growth was unaltered. However, although the increase in *c-Kit* indicated that the cells were differentiating, the expression of *Stra8*, a marker of further differentiated spermatogonia, remained low, indicating that the SSCs without GFRA1 and FGF2 were not completely differentiated, and thus were capable of proliferation *in vitro*. These results demonstrated that KSR is effective to maintain cell growth in the presence of lower cytokine concentrations in the SSCs culture medium.

SSCs derived from ICR and B6 mice can be cultured under the alternative conditions

One of the most influential factors for culturing SSCs *in vitro* is the mouse strain from which the SSCs are isolated. Previous studies demonstrated that SSCs isolated from DBA/2 mice proliferate more quickly than those from other strains such as C57BL/6 (B6), which reportedly requires GFRA1 and FGF2 for continuous proliferation [79, 80]. In fact, presumably due to a substandard batch of BSA for the SSCs culture, SSCs from non-DBA/2 strains were unable to be cultured *in vitro* under the conventional culture conditions, despite multiple attempts and increased concentrations of cytokines (data not shown). Thus, it was next tested whether the combination of KSR and BSA was applicable to SSCs from non-DBA/2 strains, including B6, ICR and 129Sv. For this experiment, all of the recommended cytokines (i.e. GDNF, GFRA1, and FGF2) were used, because a previous study demonstrated that SSCs isolated from B6 x B6:129 (i.e. 75% B6 and 25% 129Sv) mice require these cytokines for efficient proliferation [79]. As a result, although more time was required for the initiation of the proliferation, the SSCs derived from the B6 and ICR strains started to proliferate exponentially after about 4 weeks, and the doubling times between the 5th and the 7th week were calculated as 3.7 ± 0.4 days (ICR), 5.1 ± 0.3 days (C57BL/6) and 2.5 ± 0.1

days (DBA/2) (Fig. 31A). Once they started to proliferate regularly, they formed tight colonies, which were indistinguishable from those of DBA/2 (Fig. 31B), implying the effectiveness of KSR for non-DBA/2 strains. However, the SSCs from 129Sv, which are reportedly the most difficult to establish, never proliferated under this condition, and thus further modifications are required for 129Sv.

A



B

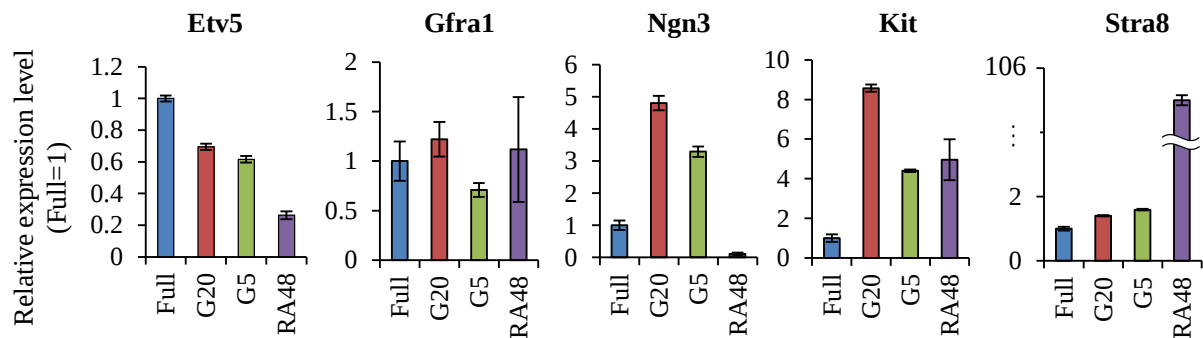


Fig. 30 GDNF is indispensable but can be reduced in the presence of KSR.

(A) Growth curves of SSCs under reduced cytokine conditions (left), summarized in the table (right panel). The number of SSCs was counted at the indicated weeks, and the cell counts are presented as means $\pm$ SD from three independent experiments. No proliferation was observed in Null condition.

(B) Relative mRNA expression levels of undifferentiated spermatogonial markers (*Gfra1*, *Etv5* and *Neurog3*) and differentiating/differentiated spermatogonial markers (*c-Kit* and *Stra8*) were examined at 12 hours after the medium change from the full cytokine conditions to the indicated conditions. RA48 cells are differentiated SSCs treated with retinoic acid for 48 hours. Data are presented as means $\pm$ SD from three independent experiments. The Y-axis indicates relative expression level to the value of Full, set to 1 for each group.

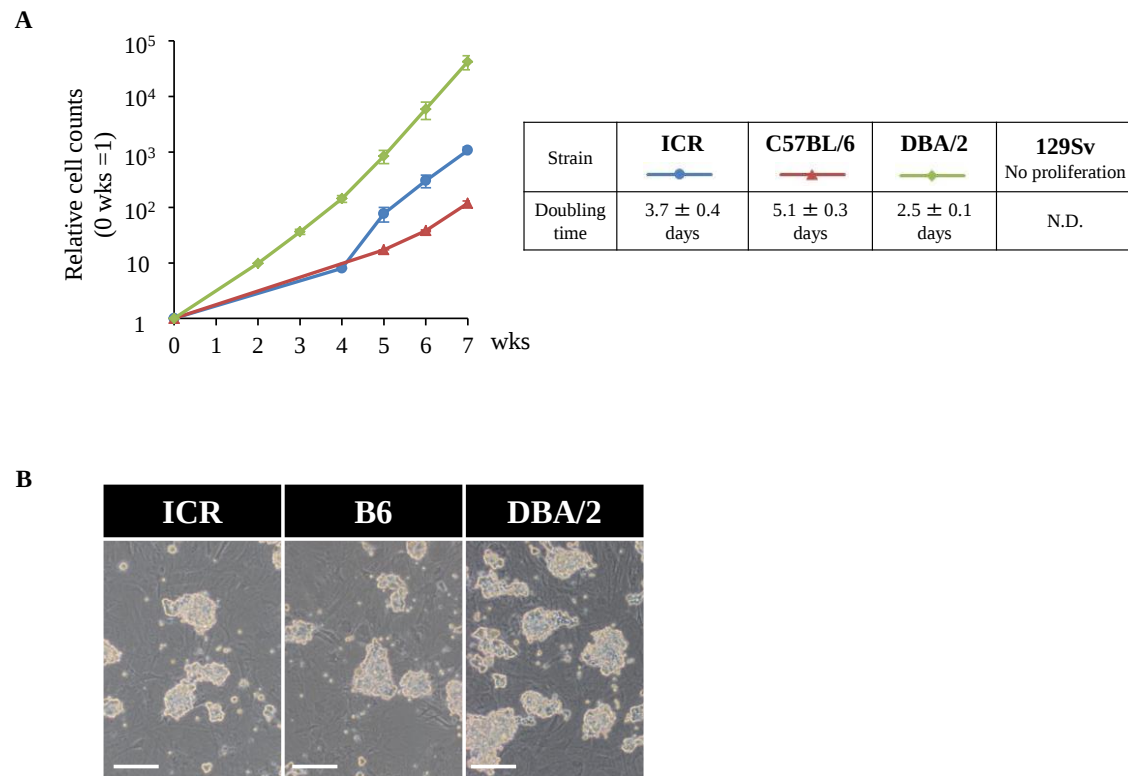


Fig. 31 SSCs derived from ICR and B6 can be cultured under the alternative conditions.

(A) Growth curves of SSCs derived from ICR, B6 and DBA/2 mice (left), summarized in the table (right). The number of SSCs was counted at the indicated time points, and cell counts are presented as means ± SD from three independent experiments. The calculated doubling times are also shown in the table (right). (B) Phase contrast images of SSCs colonies derived from ICR, B6 and DBA/2 mice. Scale bar = 100 μm.

Discussion

For cell culture, the development of serum-free, defined culture medium is beneficial to disseminate experimental methods to other laboratories with high reproducibility and reliability, since the quality of serum varies depending on the batch, the manufacturers' preparation methods, and even between individual animals. In addition, primary cells are usually sensitive to serum-containing inhibitory materials, whereas fibroblast-derived feeder cells selectively proliferate due to the growth factors within FBS. One of the major purposes for adding serum is to provide hormones or growth factors to stimulate cell replication. Therefore, supplementation of the media with optimal amounts of individual hormones and growth factors can eliminate the need for serum, without affecting the growth or other characteristics of the cells. For SSCs, previous studies reported the development of defined serum-free media by utilizing ideal cytokines, which support the long-term culture while maintaining the stemness [79, 80]. However, these reported conditions are strongly dependent on the BSA, which sometimes causes spontaneous differentiation of SSCs due to its inconsistent quality as an animal product.

In this study, it was demonstrated that KSR, a commercially available serum-free supplement, can substitute for the BSA in both *in vitro* and *in vivo* SSCs cultures without affecting their properties, such as the stem cell activity and gene expression. In addition, the combined use of KSR with BSA significantly ensures cell proliferation by facilitating cell cycle progression. KSR was initially employed for ESCs, since it prevented spontaneous differentiation, and it was subsequently utilized for other types of stem cells including human embryonic germ cells [86], suggesting its usefulness for culturing SSCs. In support of this idea, Sato *et al.* recently demonstrated that KSR is vital for testicular organ culture, and they further verified that KSR and high-quality BSA exert similar effects, suggesting that KSR is useful as an alternative for BSA for *in vitro* culture

of SSCs [82]. However, another group reported that KSR did not support the long-term culture of SSCs, when it was used with DMEM/F12 basal medium and STO feeders [87], suggesting that compatibility with the basal medium and feeder cells is also critical. Indeed, KSR along with STO feeder cells and the conventional MEM α -based medium previously reported by Kubota *et al.* were unable to be utilized [79, 80], and therefore MEF feeders were used instead (Fig. 24A). Since the conventional medium is reportedly compatible with STOs, KSR might have a detrimental effect on STOs, as observed morphological alterations of the STOs in the presence of KSR (Fig. 24B). Similarly, BSA alone was incompatible with MEFs in the absence of KSR in the culture conditions, as it caused the detachment of SSCs from MEFs (MEF_BSA, Fig. 24A). This was probably one of the major reasons why cell growth was inhibited, besides the impairment of cell cycle progression (Fig. 29). Furthermore, it is also intriguing that KSR facilitates the cell cycle progression of SSCs, because this effect hasn't been reported in ESCs and other types of stem cells.

Another remarkable finding is that even though KSR increased the growth rates of SSCs to a level close that of GSCs, the gene expression properties were still relatively similar to those of SSCs cultured under the conventional conditions. GSCs are isolated from gonocytes, which are more primitive germ cells than SSCs, and they are sometimes converted to ESC-like pluripotent stem cells that generate embryonic carcinomas [75]. This cancerous conversion would be disadvantageous for clinical usage, such as SSCs transplantation for the treatment of infertility. From this viewpoint, the qualities of KSR, which enhances the cell growth and maintains the stem cell activity simultaneously, seem to be suitable for the clinical applications of SSCs.

In addition, KSR not only allowed a significant reduction in the GDNF concentration for SSCs from the DBA/2 strain, but also was effective for SSCs from non-DBA/2 strains, except for 129Sv. Among the various mouse strains, B6 is the most widely-used strain for multiple purposes, while ICR is also popular because of its fecundity. Thus, as long as KSR is used, backcrossing to

DBA/2 is no longer required, when these two mouse strains are used to isolate and culture the SSCs. Unfortunately, however, KSR failed to support the culture of 129Sv SSCs, which are regularly used for *in vivo* gene targeting due to their high frequency of recombination. The exact reason for the strain-dependency is still unknown, but previous reports indicated that it might be caused by the sensitivity and/or dependency of the GDNF receptors. Thus, the effect of KSR is probably not due to an alteration of the GDNF pathway but to other reasons, such as osmolarity [88].

This study concluded that KSR is a reliable substitute for BSA that does not affect the properties of SSCs. This finding provides efficient and stable culture conditions for SSCs and bypasses the problems with the use of BSA, and thus will facilitate various studies using SSCs, including therapeutic applications.

Summary

Since SSCs are capable of both self-renewal and differentiation to daughter cells for subsequent spermatogenesis, the development of an efficient *in vitro* culture system is essential for studies related to spermatogenesis. Although the currently available system is serum-free and contains only chemically-defined components, it highly relies upon BSA, a component with batch-to-batch quality variations similar to those of fetal bovine serum. Thus, an alternative BSA-free culture system that preserved the properties of SSCs was searched. In this study, KSR in the SSCs culture medium was utilized as a substitute for BSA. The results demonstrated that KSR supported the continuous growth of SSCs *in vitro* and the SSCs activity *in vivo* without BSA, in a feeder-cell combination with mouse embryonic fibroblasts. The addition of BSA to KSR further facilitated cell cycle progression, whereas a transplantation assay revealed that the addition of BSA did not affect the number of SSCs *in vivo*. The combination of KSR with BSA also allowed the elimination of GFRA1 and FGF2, and the reduction of the GDNF concentration from 20 ng/ml to 5 ng/ml, while maintaining the growth rate and the expression of SSCs markers. Furthermore, KSR was also useful with SSCs from non-DBA/2 strains, such as C57BL/6 and ICR. These results suggested that KSR is an effective substitute for BSA for long-term *in vitro* cultures of SSCs. Therefore, this method is practical for various studies related to SSCs, including spermatogenesis and germ stem cell biology.

Conclusion

Reprogramming is the process that differentiated cells become to undifferentiated state by dynamic alterations of epigenetic modifications such as DNA methylations and histone modifications. It occurs naturally in zygotes soon after fertilization and primordial germ cells and it is artificially induced by nuclear transplantation into enucleated unfertilized oocytes or gene transfection of defined transcription factors. Although reprogramming is induced by artificially, the success rate is much lower than the natural one and the difference of molecular mechanisms between natural and artificial is also unclear. Elucidating molecular mechanisms of reprogramming cannot only provide new insights into basic life sciences such as molecular biology and embryology but also promote the development of regenerative medicines by improving the reprogramming efficiency. I studied reprogramming in germ cells to reveal the molecular mechanism.

In chapter1, the roles of histone modifications during reprogramming occurring at the PN stage soon after fertilization were examined. Although histone modifications are altered in the paternal pronucleus at the PN stage, the most important histone modification for the early embryonic development has not yet been determined. In this study, the roles of histone methylations were studied by abrogating global histone methylations with K-M mutants, which prevent endogenous histone methylation at the mutated site. Four K-M mutants (H3.3 K4M, K9M, K27M and K36M) at the sites required for transcription were used and H3.3 K4M overexpression demonstrated that H3K4 methylation is required for minor ZGA and subsequent early embryonic development. Furthermore, knockdown of *Mll3* and *Mll4*, H3K4 methyltransferases, indicated same results as the overexpression of H3.3 K4M. These results suggested that H3K4 methylation, likely established by Mll3/4 at the early PN stage, is essential for the onset of minor ZGA in the paternal pronucleus and subsequent early embryonic development in reprogramming at the PN stage in mice.

In chapter 2, availability of KSR and MEFs for SSCs culture was examined to establish

effective culture method of SSCs. Previous culture methods required BSA, an animal product with quality variations depending on manufactures or lots, which made it difficult to maintain reproducible culture conditions. As a recent report has demonstrated that KSR was successfully used for testicular organ culture *in vitro* without serum and cytokines, I examined whether KSR can be also used for SSCs culture instead of BSA. SSCs cultured with KSR showed high proliferation activity without BSA, and furthermore, transplantation of SSCs under the condition revealed that they retained stemness, which indicated that KSR can be used as a substitute for BSA in SSCs culture. Adding both KSR and BSA showed higher proliferation activity compared to only KSR, could remove GFRA1 and FGF2 and reduced the GDNF concentration from 20 ng/ml to 5 ng/ml. Although previous cultured methods can support SSCs growth only derived from DBA/2 strain mice, combination of KSR and BSA can make it possible to culture SSCs derived from ICR and B6 strains. These results suggest that KSR can make SSCs culture effectively and facilitate studies on SSCs derived from various mouse strains.

In this study, the role of histone methylation in reprogramming at the PN stage in mice was revealed and efficient SSCs culture was established. These results should contribute to elucidation of the molecular mechanisms of reprogramming in mouse germ cells and improvement of the artificial reprogramming efficiency.

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References

1. **Reik W, Dean W, Walter J.** 2001. Epigenetic reprogramming in mammalian development. *Science* **293**: 1089-1093
2. **Leitch HG, Tang WW, Surani MA.** 2013. Primordial germ-cell development and epigenetic reprogramming in mammals. *Curr Top Dev Biol* **104**: 149-187
3. **Gurdon JB, Elsdale TR, Fischberg M.** 1958. Sexually mature individuals of *Xenopus laevis* from the transplantation of single somatic nuclei. *Nature* **182**: 64-65
4. **Campbell KH, McWhir J, Ritchie WA, Wilmut I.** 1996. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**: 64-66
5. **Takahashi K, Yamanaka S.** 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**: 663-676
6. **Bird A.** 2002. DNA methylation patterns and epigenetic memory. *Genes Dev* **16**: 6-21
7. **Kouzarides T.** 2007. Chromatin modifications and their function. *Cell* **128**: 693-705
8. **Sarma K, Reinberg D.** 2005. Histone variants meet their match. *Nat Rev Mol Cell Biol* **6**: 139-149
9. **Probst AV, Dunleavy E, Almouzni G.** 2009. Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* **10**: 192-206
10. **Lee JH, Hart SR, Skalnik DG.** 2004. Histone deacetylase activity is required for embryonic stem cell differentiation. *Genesis* **38**: 32-38
11. **Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES.** 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**: 315-326
12. **Gan Q, Yoshida T, McDonald OG, Owens GK.** 2007. Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. *Stem Cells* **25**: 2-9
13. **Meissner A.** 2010. Epigenetic modifications in pluripotent and differentiated cells. *Nat Biotechnol* **28**: 1079-1088
14. **Chou BK, Mali P, Huang X, Ye Z, Dowey SN, Resar LM, Zou C, Zhang YA, Tong J, Cheng L.** 2011. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res* **21**: 518-529
15. **Nazor KL, Altun G, Lynch C, Tran H, Harness JV, Slavin I, Garitaonandia I, Muller FJ, Wang YC, Boscolo FS, Fakunle E, Dumevska B, Lee S, Park HS, Olee T, D'Lima DD, Semechkin R, Parast MM, Galat V, Laslett AL, Schmidt U, Keirstead HS, Loring JF, Laurent LC.** 2012. Recurrent variations in DNA methylation in human pluripotent stem cells

- and their differentiated derivatives. *Cell Stem Cell* **10**: 620-634
16. **Yang BH, Floess S, Hagemann S, Deyneko IV, Groebe L, Pezoldt J, Sparwasser T, Lochner M, Huehn J.** 2015. Development of a unique epigenetic signature during in vivo Th17 differentiation. *Nucleic Acids Res* **43**: 1537-1548
 17. **Morgan HD, Santos F, Green K, Dean W, Reik W.** 2005. Epigenetic reprogramming in mammals. *Hum Mol Genet* **14 Spec No 1**: R47-58
 18. **Cantone I, Fisher AG.** 2013. Epigenetic programming and reprogramming during development. *Nat Struct Mol Biol* **20**: 282-289
 19. **Takahashi K, Yamanaka S.** 2015. A developmental framework for induced pluripotency. *Development* **142**: 3274-3285
 20. **Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T.** 2007. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat Genet* **39**: 295-302
 21. **Adenot PG, Mercier Y, Renard JP, Thompson EM.** 1997. Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. *Development* **124**: 4615-4625
 22. **Santos F, Hendrich B, Reik W, Dean W.** 2002. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol* **241**: 172-182
 23. **Arney KL, Bao S, Bannister AJ, Kouzarides T, Surani MA.** 2002. Histone methylation defines epigenetic asymmetry in the mouse zygote. *Int J Dev Biol* **46**: 317-320
 24. **Lepikhov K, Walter J.** 2004. Differential dynamics of histone H3 methylation at positions K4 and K9 in the mouse zygote. *BMC Dev Biol* **4**: 12
 25. **Torres-Padilla ME, Bannister AJ, Hurd PJ, Kouzarides T, Zernicka-Goetz M.** 2006. Dynamic distribution of the replacement histone variant H3.3 in the mouse oocyte and preimplantation embryos. *Int J Dev Biol* **50**: 455-461
 26. **Santenard A, Ziegler-Birling C, Koch M, Tora L, Bannister AJ, Torres-Padilla ME.** 2010. Heterochromatin formation in the mouse embryo requires critical residues of the histone variant H3.3. *Nat Cell Biol* **12**: 853-862
 27. **Boskovic A, Bender A, Gall L, Ziegler-Birling C, Beaujean N, Torres-Padilla ME.** 2012. Analysis of active chromatin modifications in early mammalian embryos reveals uncoupling of H2A.Z acetylation and H3K36 trimethylation from embryonic genome activation. *Epigenetics* **7**: 747-757
 28. **Gu TP, Guo F, Yang H, Wu HP, Xu GF, Liu W, Xie ZG, Shi L, He X, Jin SG, Iqbal K, Shi YG, Deng Z, Szabo PE, Pfeifer GP, Li J, Xu GL.** 2011. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature* **477**: 606-610
 29. **Nakamura T, Liu YJ, Nakashima H, Umehara H, Inoue K, Matoba S, Tachibana M,**

- Ogura A, Shinkai Y, Nakano T.** 2012. PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. *Nature* **486**: 415-419
30. **van der Heijden GW, Dieker JW, Derijck AA, Muller S, Berden JH, Braat DD, van der Vlag J, de Boer P.** 2005. Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote. *Mech Dev* **122**: 1008-1022
31. **Lin CJ, Conti M, Ramalho-Santos M.** 2013. Histone variant H3.3 maintains a decondensed chromatin state essential for mouse preimplantation development. *Development* **140**: 3624-3634
32. **Golbus MS, Calarco PG, Epstein CJ.** 1973. The effects of inhibitors of RNA synthesis (alpha-amanitin and actinomycin D) on preimplantation mouse embryogenesis. *J Exp Zool* **186**: 207-216
33. **Warner CM, Versteegh LR.** 1974. In vivo and in vitro effect of alpha-amanitin on preimplantation mouse embryo RNA polymerase. *Nature* **248**: 678-680
34. **Latham KE, Garrels JI, Chang C, Solter D.** 1991. Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages. *Development* **112**: 921-932
35. **Bouniol C, Nguyen E, Debey P.** 1995. Endogenous transcription occurs at the 1-cell stage in the mouse embryo. *Exp Cell Res* **218**: 57-62
36. **Aoki F, Worrall DM, Schultz RM.** 1997. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev Biol* **181**: 296-307
37. **Ong CT, Corces VG.** 2011. Enhancer function: new insights into the regulation of tissue-specific gene expression. *Nat Rev Genet* **12**: 283-293
38. **Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, Ye Z, Lee LK, Stuart RK, Ching CW, Ching KA, Antosiewicz-Bourget JE, Liu H, Zhang X, Green RD, Lobanov VV, Stewart R, Thomson JA, Crawford GE, Kellis M, Ren B.** 2009. Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* **459**: 108-112
39. **Rada-Iglesias A, Bajpai R, Swigut T, Brugmann SA, Flynn RA, Wysocka J.** 2011. A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* **470**: 279-283
40. **Calo E, Wysocka J.** 2013. Modification of enhancer chromatin: what, how, and why? *Mol Cell* **49**: 825-837
41. **Hu D, Gao X, Morgan MA, Herz HM, Smith ER, Shilatifard A.** 2013. The MLL3/MLL4 branch of the COMPASS family is a major H3K4 monomethylase at enhancers. *Mol Cell Biol*, 10.1128/MCB.01181-13
42. **Cheng J, Blum R, Bowman C, Hu D, Shilatifard A, Shen S, Dynlacht BD.** 2014. A role for H3K4 monomethylation in gene repression and partitioning of chromatin readers. *Mol Cell* **53**:

979-992

43. **Mohan M, Herz HM, Smith ER, Zhang Y, Jackson J, Washburn MP, Florens L, Eissenberg JC, Shilatifard A.** 2011. The COMPASS family of H3K4 methylases in *Drosophila*. *Mol Cell Biol* **31**: 4310-4318
44. **Herz HM, Mohan M, Garruss AS, Liang K, Takahashi YH, Mickey K, Voets O, Verrijzer CP, Shilatifard A.** 2012. Enhancer-associated H3K4 monomethylation by Trithorax-related, the *Drosophila* homolog of mammalian Mll3/Mll4. *Genes Dev* **26**: 2604-2620
45. **Lee JE, Wang C, Xu S, Cho YW, Wang L, Feng X, Baldridge A, Sartorelli V, Zhuang L, Peng W, Ge K.** 2013. H3K4 mono- and di-methyltransferase MLL4 is required for enhancer activation during cell differentiation. *Elife* **2**: e01503
46. **Wang S, Kou Z, Jing Z, Zhang Y, Guo X, Dong M, Wilmot I, Gao S.** 2010. Proteome of mouse oocytes at different developmental stages. *Proc Natl Acad Sci U S A* **107**: 17639-17644
47. **Lewis PW, Muller MM, Koletsky MS, Cordero F, Lin S, Banaszynski LA, Garcia BA, Muir TW, Becher OJ, Allis CD.** 2013. Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. *Science* **340**: 857-861
48. **Miki H, Ogonuki N, Inoue K, Baba T, Ogura A.** 2006. Improvement of cumulus-free oocyte maturation in vitro and its application to microinsemination with primary spermatocytes in mice. *J Reprod Dev* **52**: 239-248
49. **Nagy A.** 2003. *Manipulating the mouse embryo : a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Tagami H, Ray-Gallet D, Almouzni G, Nakatani Y.** 2004. Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* **116**: 51-61
51. **Yamagata K, Yamazaki T, Yamashita M, Hara Y, Ogonuki N, Ogura A.** 2005. Noninvasive visualization of molecular events in the mammalian zygote. *Genesis* **43**: 71-79
52. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: RESEARCH0034
53. **Mamo S, Gal AB, Bodo S, Dinnyes A.** 2007. Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured in vivo and in vitro. *BMC Dev Biol* **7**: 14
54. **Rasband WS.** 1997-2014. ImageJ. *Journal*, <http://imagej.nih.gov/ij/>
55. **Abràmoff MD, Magalhães PJ, Ram SJ.** 2004. Image processing with ImageJ. *Biophotonics international* **11**: 36-43
56. **Schneider CA, Rasband WS, Eliceiri KW.** 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**: 671-675

57. **Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R.** 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**: 369-374
58. **Paull D, Emmanuele V, Weiss KA, Treff N, Stewart L, Hua H, Zimmer M, Kahler DJ, Goland RS, Noggle SA, Prosser R, Hirano M, Sauer MV, Egli D.** 2013. Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants. *Nature* **493**: 632-637
59. **Akiyama T, Suzuki O, Matsuda J, Aoki F.** 2011. Dynamic replacement of histone H3 variants reprograms epigenetic marks in early mouse embryos. *PLoS Genet* **7**: e1002279
60. **Makino Y, Inoue E, Hada M, Aoshima K, Kitano S, Miyachi H, Okada Y.** 2014. Generation of a dual-color reporter mouse line to monitor spermatogenesis in vivo. *Front Cell Dev Biol* **2**: 30
61. **Eissenberg JC, Shilatifard A.** 2010. Histone H3 lysine 4 (H3K4) methylation in development and differentiation. *Dev Biol* **339**: 240-249
62. **Smith E, Lin C, Shilatifard A.** 2011. The super elongation complex (SEC) and MLL in development and disease. *Genes Dev* **25**: 661-672
63. **Deb M, Kar S, Sengupta D, Shilpi A, Parbin S, Rath SK, Londhe VA, Patra SK.** 2014. Chromatin dynamics: H3K4 methylation and H3 variant replacement during development and in cancer. *Cell Mol Life Sci*, 10.1007/s00018-014-1605-4
64. **Park SJ, Komata M, Inoue F, Yamada K, Nakai K, Ohsugi M, Shirahige K.** 2013. Inferring the choreography of parental genomes during fertilization from ultralarge-scale whole-transcriptome analysis. *Genes Dev* **27**: 2736-2748
65. **Park SJ, Shirahige K, Ohsugi M, Nakai K.** 2015. DBTMEE: a database of transcriptome in mouse early embryos. *Nucleic Acids Res* **43**: D771-776
66. **Shilatifard A.** 2012. The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. *Annu Rev Biochem* **81**: 65-95
67. **Okada Y, Yamagata K, Hong K, Wakayama T, Zhang Y.** 2010. A role for the elongator complex in zygotic paternal genome demethylation. *Nature* **463**: 554-558
68. **Liu W, Yin J, Kou X, Jiang Y, Gao H, Zhao Y, Huang B, He W, Wang H, Han Z, Gao S.** 2014. Asymmetric reprogramming capacity of parental pronuclei in mouse zygotes. *Cell Rep* **6**: 1008-1016
69. **Tokoro M, Shin SW, Nishikawa S, Lee HH, Hatanaka Y, Amano T, Mitani T, Kato H, Anzai M, Kishigami S, Saeki K, Hosoi Y, Iritani A, Matsumoto K.** 2010. Deposition of acetylated histones by RNAP II promoter clearance may occur at onset of zygotic gene activation in preimplantation mouse embryos. *J Reprod Dev* **56**: 607-615
70. **Ram PT, Schultz RM.** 1993. Reporter gene expression in G2 of the 1-cell mouse embryo. *Dev Biol* **156**: 552-556

71. **Andreu-Vieyra CV, Chen R, Agno JE, Glaser S, Anastassiadis K, Stewart AF, Matzuk MM.** 2010. MLL2 is required in oocytes for bulk histone 3 lysine 4 trimethylation and transcriptional silencing. *PLoS Biol* **8**:
72. **Bultman SJ, Gebuhr TC, Pan H, Svoboda P, Schultz RM, Magnuson T.** 2006. Maternal BRG1 regulates zygotic genome activation in the mouse. *Genes Dev* **20**: 1744-1754
73. **Torres-Padilla ME, Zernicka-Goetz M.** 2006. Role of TIF1alpha as a modulator of embryonic transcription in the mouse zygote. *J Cell Biol* **174**: 329-338
74. **Hamatani T, Carter MG, Sharov AA, Ko MS.** 2004. Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell* **6**: 117-131
75. **Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A, Shinohara T.** 2004. Generation of pluripotent stem cells from neonatal mouse testis. *Cell* **119**: 1001-1012
76. **Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G.** 2006. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* **440**: 1199-1203
77. **Ko K, Tapia N, Wu G, Kim JB, Bravo MJ, Sasse P, Glaser T, Ruau D, Han DW, Greber B, Hausdorfer K, Sebastiano V, Stehling M, Fleischmann BK, Brustle O, Zenke M, Scholer HR.** 2009. Induction of pluripotency in adult unipotent germline stem cells. *Cell Stem Cell* **5**: 87-96
78. **Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL.** 1998. Culture of mouse spermatogonial stem cells. *Tissue Cell* **30**: 389-397
79. **Kubota H, Avarbock MR, Brinster RL.** 2004. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci U S A* **101**: 16489-16494
80. **Kubota H, Brinster RL.** 2008. Culture of rodent spermatogonial stem cells, male germline stem cells of the postnatal animal. *Methods Cell Biol* **86**: 59-84
81. **Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T.** 2003. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod* **69**: 612-616
82. **Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T.** 2011. In vitro production of functional sperm in cultured neonatal mouse testes. *Nature* **471**: 504-507
83. **Kubota H, Avarbock MR, Brinster RL.** 2003. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proc Natl Acad Sci U S A* **100**: 6487-6492
84. **Yamaguchi S, Kimura H, Tada M, Nakatsuji N, Tada T.** 2005. Nanog expression in mouse germ cell development. *Gene Expr Patterns* **5**: 639-646

85. **Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP.** 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet* **36**: 653-659
86. **Hua J, Yu H, Liu S, Dou Z, Sun Y, Jing X, Yang C, Lei A, Wang H, Gao Z.** 2009. Derivation and characterization of human embryonic germ cells: serum-free culture and differentiation potential. *Reprod Biomed Online* **19**: 238-249
87. **Wang QZ, Hna CS.** 2008. [Serum-free knockout SR medium supports the short-time viability of mouse spermatogonial stem cells]. *Fen Zi Xi Bao Sheng Wu Xue Bao* **41**: 162-166
88. **Rajala K, Vaajasaari H, Suuronen R, Hovatta O, Skottman H.** 2011. Effects of the physiochemical culture environment on the stemness and pluripotency of human embryonic stem cells. *Stem Cell Studies* **1**:

Summary in Japanese

リプログラミングとは DNA メチル化やヒストン修飾などのエピジェネティック修飾が大きく変化することにより，一度分化した細胞が未分化状態に戻る機構である。自然界では受精直後の受精卵や始原生殖細胞において，人工的には未受精卵への核移植や転写因子の遺伝子導入により引き起こされる。リプログラミングは現象としては人工的に再現できるものの，その効率は自然界のものに比べて著しく低く，両者の分子機構の違いも明らかではない。リプログラミングの分子機構の解明は分子生物学や発生学といった基礎生命科学に新規の知見をもたらすだけでなく，リプログラミング効率の向上を可能にし，再生医療の発展をより一層推し進めることができる。そこで，生殖細胞に生じるリプログラミングに着目し，その分子機構を明らかにすることを目的とし，研究を行った。

第一章では受精直後の受精卵前核期に生じるリプログラミングにおけるヒストン修飾の役割を調べた。前核期では特に雄性前核においてヒストン修飾が大きく変化することが知られているが，どのヒストン修飾が初期胚発生に最も重要であるかはわかっていない。本研究では転写に重要なヒストンメチル化修飾 (H3K4, K9, K27, K36) に焦点を絞り，それぞれのリジン残基をメチオニン残基に変換した変異体 (K-M 変異体) を用いて当該部位の内在性メチル化修飾を消去することで，前核期におけるヒストンメチル化修飾の役割を調べた。その結果，雄性前核における H3K4 メチル化が胚性ゲノム活性化 (minor zygotic gene activation: minor ZGA) とその後の初期胚発生に必須であることがわかった。更に，H3K4 メチル化酵素のうち，Mll3/4 をノックダウンした時に H3K4 メチル化修飾を消去した場合と同様の結果が認められた。以上の結果から，マウス受精卵前核期に生じるリプログラミングにおいて，雄性前核において Mll3/4 により成立する H3K4 メチル化が雄性前核の minor ZGA の開始と初期胚発生に必須であることがわかった。

第二章では精原幹細胞 (Spermatogonial stem cells: SSCs) の効率的な培養方法の開発を目

的として Knockout Serum Replacement (KSR) と Mouse embryonic fibroblast (MEF) の有用性を調べた。従来の培養法では Bovine serum albumin (BSA) の添加が必須であったが、動物由来の BSA はロット間や製造者間でのばらつきがあり、再現性のある培養条件を保つことが難しかった。近年、血清代替産物である KSR を用いることで血清やサイトカインを用いずに精巣の臓器培養が可能であることが報告された。そこで、KSR が BSA の代替物として SSCs の培養にも適用できるかどうかを調べた。その結果、KSR を添加すると BSA 非添加の条件でも SSCs は高い増殖活性を示し、ヌードマウスへの移植実験においても幹細胞性を保持していることが確認されたため、KSR が BSA の代替物として利用可能であることがわかった。また、KSR と BSA を同時に添加することで、BSA 非添加条件と比べて高い増殖活性を示した。更に従来の培養法では三種類のサイトカイン (Glial cell line-derived neurotrophic factor: GDNF, GDNF family receptor alpha 1: GFRA1, Fibrous growth factor 2: FGF2) の添加が必要であったが、GDNF のみで SSCs の培養が可能となり、必要な GDNF 量も 20 ng/ml から 5 ng/ml まで減らすことができた。これまでの培養法では DBA/2 マウス由来の SSCs しか培養できなかったが、KSR と BSA を添加した条件下では ICR マウスと C57BL/6 マウス由来の SSCs も培養可能となった。以上から、KSR を添加することで SSCs の培養がより効率的になり、様々なマウス由来の SSCs を用いた研究を行うことが可能となった。

本研究によりマウス受精卵前核期リプログラミング機構の一端が解明されるとともに、マウス SSCs の効率的な培養が可能となった。本研究の知見はマウス生殖細胞におけるリプログラミング機構の解明に貢献するものであり、人為的リプログラミングの更なる効率化にも資することが期待される。